Development of a Chemiluminescence Competitive PCR for the Detection and Quantification of Parvovirus B19 DNA Using a Microplate Luminometer

Fabiana Fini,1 Giorgio Gallinella,2 Stefano Girotti,1 Marialuisa Zerbini,2 and Monica Musiani2*

Background: Quantitative PCR of viral nucleic acids can be useful clinically in diagnosis, risk assessment, and monitoring of antiviral therapy. We wished to develop a chemiluminescence competitive PCR (cPCR) for parvovirus B19.

Methods: Parvovirus DNA target sequences and competitor sequences were coamplified and directly labeled. Amplified products were then separately hybridized by specific biotin-labeled probes, captured onto streptavidin-coated ELISA microplates, and detected immunoenzymatically using chemiluminescent substrates of peroxidase. Chemiluminescent signals were quantitatively analyzed by a microplate luminometer and were correlated to the amounts of amplified products.

Results: Luminol-based systems displayed constant emission but had a higher detection limit (100–1000 genome copies) than the acridan-based system (20 genome copies). The detection limit of chemiluminescent substrates was lower (20 genome copies) than colorimetric substrates (50 genome copies). In chemiluminescence cPCR, the titration curves showed linear correlation above 100 target genome copies. Chemiluminescence cPCR was positive in six serum samples from patients with parvovirus infections and negative in six control sera.

Conclusions: The chemiluminescence cPCR appears to be a sensitive and specific method for the quantitative detection of viral DNAs.

© 1999 American Association for Clinical Chemistry

PCR, with its extraordinary sensitivity, is the method of choice for the detection of nucleic acids present in very low concentrations in biological specimens. The need for quantitative determination of PCR products led at first to the development of several semiquantitative methods such as those based on limiting dilution of the analyte (1), on external calibration curves for quantification (2), or on low stringency PCR (3). More recently, absolute quantitative methods involving an internally controlled amplification reaction such as competitive PCR (cPCR)3 have been developed (4, 5).

cPCR is based on the coamplification of the target sequence to be quantified and of a competitor sequence that is introduced in a known amount. The competitor sequence must be as similar as possible to the target sequence but easily discriminable from it. To date, procedures such as hybridization followed by colorimetric immunoenzymatic detection (6), gel electrophoresis (7), or capillary electrophoresis (8) have been used to differentiate and quantify the amplification products.

In the study of infectious diseases, these quantitative cPCR assays can be very useful in understanding disease progression, in monitoring antiviral therapy, and in evaluating the potential risks of transmission of pathogens. Moreover, they can be very useful in diagnosing infectious diseases that can persist in the presence of a small number of infectious agents and in distinguishing low-level, innocuous infections from those that may be of clinical relevance.

In a recent study, we developed a cPCR that used

1 Institute of Chemical Science and 2 Department of Clinical and Experimental Medicine, Division of Microbiology, University of Bologna, Via Masantini 9, 40138 Bologna, Italy.
*Author for correspondence. Fax 0039-051-341632; e-mail musiani@med.unibo.it.
Received February 18, 1999; accepted June 4, 1999.

3 Nonstandard abbreviations: cPCR, competitive PCR; nt, nucleotide(s); TBST, Tris-buffered saline plus Tween (150 mmol/L NaCl, 100 mmol/L Tris HCl, pH 7.5, 1 mL/L Tween 20); HRP, horseradish peroxidase; SS, SuperSignal; SS-Ultra, SuperSignal ULTRA; and RLU, relative light unit(s).
colorimetric immunoenzymatic detection for the quantification of B19 parvovirus genomes (9). B19 parvovirus, in fact, is a widespread virus that can cause acute diseases, such as erythema infectiosum, fetal hydrops, postinfectious arthropathy, and transient aplastic crises; it also can cause chronic infections that can occur with very low blood viral titers (10).

In recent years, chemiluminescent reactions have been successfully used for the sensitive and semiquantitative detection of PCR-amplified products (11–13); moreover, the research and synthesis of new chemiluminescent substrates has been matched with new developments in chemiluminescence instrumentation such as ultrasensitive luminographs (14, 15) and microplate luminometers (13, 16). The aim of the present study, therefore, was to develop a chemiluminescence cPCR for the detection and absolute quantification of low concentrations of viral DNAs, using parvovirus B19 DNA as a model, and to evaluate its potential for diagnostic purposes.

The quantitative chemiluminescence cPCR described here was based on the coamplification of parvovirus DNA target original sequences and of competitor sequences differing from the original sequence in a 21-bp mutated sequence. In our assay, both target and competitor were amplified by the same set of primers and labeled during amplification with digoxigenin-labeled dUTP (17). The amplified products were then hybridized in two separate reactions with biotin-labeled probes specific for either the target or competitor mutant sequence. Hybridized amplicons were then captured onto streptavidin-coated ELISA plates and detected by anti-digoxigenin antibodies conjugated to peroxidase, using a chemiluminescent substrate. Chemiluminescent signals were quantitatively analyzed by a microplate luminometer and were correlated to the amounts of amplified products.

The activity of different chemiluminescent substrates for peroxidase was evaluated by comparing the enhanced luminol system (18, 19) with a new system that uses an acridan as emitting molecule (20) according to the scheme:

$$\text{HRP, H}_2\text{O}_2 \xrightarrow{\text{Buffer, H}_2\text{O}_2} \text{N-Methylacridone}^*$$

The potential of chemiluminescence cPCR for diagnostic purposes was analyzed in positive and negative reference clinical samples.

**Materials and Methods**

**SAMPLES**
The target DNA sequence used to develop the chemiluminescence cPCR consisted of full-length B19 DNA genome cloned in plasmid pUC18 (21) and amplified in *Escherichia coli* DH5α. Plasmids were prepared by routine methods (22). The concentrations of serial 10-fold dilutions of plasmid DNA were determined by ultraviolet absorbance at 260 nm and DNA Dipstick (Invitrogen). Serial 10-fold dilutions were used as calibrators for genome copy number. All samples were diluted in 50 mg/L herring sperm DNA in Tris-EDTA buffer (10 mmol/L Tris HCl, pH 7.5, 1 mmol/L EDTA).

The competitor sequence consisted of a B19 DNA sequence of 721 bp [nucleotides (nt) 1419–2139], mutated by recombinant PCR in an internal sequence of 21 bp (nt 1733–1753) (9). The mutated sequence matched the following criteria: (a) it was unique in B19 DNA genome; (b) the base composition was unchanged; and (c) there were minor changes in thermal stability. The competitor sequence was cloned in plasmid pUC19 (Roche Molecular Biochemicals), amplified in *E. coli* DH5α, and processed as described above. Blank samples consisted of 50 mg/L herring sperm DNA in Tris-EDTA buffer.

Six reference serum samples taken from patients in early convalescent phase and patients with chronic infections positive for B19 DNA by nested PCR (23, 24) and six serum samples obtained from persons without serological clinical evidence of B19 infection and negative for B19 DNA by nested PCR were tested by chemiluminescence cPCR.

**cPCR DESIGN**

For coamplification of both target and competitor DNA, a B19 parvovirus DNA internal sequence of 184 bp corresponding to nt 1652–1835 was chosen. The oligonucleotide primers used in the amplification reaction were 5′-CTGGAGTACCTGTGGTTA-3′ (nt 1652–1669; *T_m* 39.1 °C) and 5′-CACCATGTAAAGCCACTGT-3′ (nt 1835–1818; *T_m* 42.7 °C).

Target and competitor sequences were then recognized by probe T (5′-AAGCTTAAAGAGCGAATGG-3′; *T_m* 49.9 °C), which was specific for the target original 21-bp sequence, and probe C (5′-GGATTCCGAAGAGATAG-3′; *T_m* 52.6 °C), which was specific for the competitor mutated sequence. Both probes T and C were biotin-labeled at the 5′ end.

**cPCR AMPLIFICATION**
The cPCR titration curve was determined by coamplification of a constant amount of target DNA with increasing amounts of competitor DNA. The reaction was carried out as follows: 1 μL of both target and competitor B19 DNA, at the different concentrations tested, was added to a reaction mixture (50-μL final volume) containing 50 mmol/L KCl; 2.5 mmol/L MgCl₂; 10 mmol/L Tris HCl, pH 9.0; 0.1 mmol/L dATP, dGTP, and dCTP; 0.095 mmol/L dTTP; 0.005 mmol/L digoxigenin-UTP; 0.1
μmol/L amplification primers; and 2 U of Taq DNA polymerase (Roche). After an initial denaturation step at 95 °C for 5 min, 40 cycles were performed under the following conditions: 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min, followed by an extension step at 72 °C for 5 min.

HYBRIDIZATION OF AMPLICONS WITH BIOTIN-LABELED PROBES AND CAPTURE ON MICROPLATE
For each sample, the following reactions were performed in duplicate, using probe T, specific for the original target B19 sequence, and probe C, specific for the competitor mutated sequence.

A volume of 10 μL of amplification reaction was added to 10 μL of denaturing solution (100 mmol/L NaOH, 10 mL Tween 20) and incubated at 25 °C for 10 min, after which 200 μL of hybridization solution (300 mmol/L NaCl, 100 mmol/L Tris HCl, pH 6.5, 10 mmol/L EDTA, 1.0 mL/L Tween 20) containing either probe T or probe C at the optimized concentration of 10 nmol/L was added. Hybridization was performed at 50 °C for 10 min. After hybridization, 200 μL of the hybridization mixture was transferred to a black streptavidin-coated microtiter plate well and incubated at 25 °C for 30 min to allow the capture of biotinylated probes. Plates were then washed five times with Tris-buffered saline-Tween (TBST; 150 mmol/L NaCl, 100 mmol/L Tris HCl, pH 7.5, 1 mL/L Tween 20).

CHEMILUMINESCENT DETECTION OF DIGOXIGENIN-LABELED AMPLICONS
To detect digoxigenin-labeled amplicons hybridized with biotinylated probes, 200 μL of anti-digoxigenin-horseradish peroxidase (HRP)-conjugated antibody (10 U/L in TBST; Roche Molecular) was added and incubated at 25 °C for 30 min to allow the capture of biotinylated probes. Plates were then washed five times with TBST.

Several chemiluminescent substrates for HRP were tested: ECL and ECL Plus (Amersham Life Sciences), SuperSignal (SS) and SuperSignal ULTRA (SS-Ultra; Pierce). Three substrates (ECL, SS, and SS-Ultra) were based on the acridan system (3,2′,3′,6′-trifluorophenyl-10-methylacridan-9-carboxylate).

The selected chemiluminescent substrates (100 μL) were prepared according to the manufacturers’ instructions. The chemiluminescent reaction was started by adding the substrate to each well. The emitted light was measured at room temperature in the colorimetric-luminometer device Victor 1420 (Wallac) with acquisition time set at 5 s, and the signal was expressed in relative light units (RLU) per second.

DATA ANALYSIS
For the chemiluminescent cPCR titration curve, amplification reactions involving a constant amount of target DNA genome copies and increasing amounts of the competitor DNA genome copies were performed. For each amplification reaction, the difference in RLU values between competitor and target amplicons was calculated. These were plotted against the competitor genome copy number. Linear interpolation was performed to obtain a value with a difference in RLU emission of 0. This interpolated value at the point of equivalence is the best estimate of target genome copy number present in the test sample (9). All data were analyzed using Excel software (Microsoft).

RESULTS
To optimize the performance of the chemiluminescence cPCR assay, we used the ECL, ECL Plus, SS, and SS-Ultra substrates for HRP and studied their kinetics in a standard model of single PCR using 1000 B19 target genome copies. The kinetics were measured three times for each substrate. The kinetics for each substrate are shown in Fig. 1. ECL Plus reached maximum emission (expressed in RLU) after 2 min, plateaued for ~2–4 min, and then slowly decreased; ECL, SS, and SS-Ultra, however, reached a maximum emission within seconds after addition, and the signal remaining constant for at least 10–20 min. In our system, the reproducibility of SS-Ultra emission was low, and the SD was ~10–20%. To determine whether the kinetics of the four substrates could be influenced by different amounts of target DNA, we evaluated them with 104 and 106 genome copies; the RLU profiles for both concentrations were comparable to those described above. Thereafter, the emission was measured after 4 min for ECL Plus and after 10 s for ECL, SS, and SS-Ultra.

ECL Plus, ECL, SS, and SS-Ultra were then used in different single PCRs performed in the same assay with different amounts of target DNA (1 to 106 genome copies), and the results are shown in Fig. 2. In comparison with the other substrates, ECL Plus showed a higher sensitivity because it presented a higher variation in the signal in relation to increasing amounts of target genome copies. The background emission for SS and SS-Ultra was constantly higher than for ECL and ECL Plus; ECL Plus gave the best signal-to-noise ratio (202 for 106 genome copies compared with 32 for ECL, 10 for SS-Ultra, and 7 for SS). ECL Plus was able to detect ~10–100 target genome copies, whereas ECL, SS, SS-Ultra detected ~100–1000 genome copies. These data prompted us to choose ECL Plus for subsequent PCR experiments.

To develop chemiluminescent competitive titration curves, a set of amplification reactions was carried out. Different amounts of target and competitor sequences (1 to 106 genome copies) were amplified separately and hybridized using the respective probes, and the hybrids were revealed immunoenzymatically. The detection limit of the chemiluminescence PCR (defined as the genome copy number that produced an emission 3 SD above background) was 20 copies of both target and competitor sequences. The CV and signal-to-noise ratio at the detection limit were 9.1% and 3.1, respectively. Chemilumines-
cence detection was then compared with colorimetric detection under the same experimental conditions and in the same assays. The detection limit of the colorimetric assay, performed as described previously using the ABTS [2,2'-azino-dil(3-ethyl-benzthiazoline)] enzymatic substrate (9), was 50 copies of both target and competitor sequences (Fig. 3). The CV and signal-to-noise ratio at the detection limit were 3.5% and 3.1, respectively.

Once the sensitivity of chemiluminescence PCR in single determinations had been established, constant amounts of target sequences (10–500 genome copies) were coamplified with increasing amounts of competitor sequences (10–1000 copies) and analyzed by chemiluminescence to obtain titration curves. For each titration curve, the differences between the emission values of the competitor and target amplification products were plotted against competitor copy number. The interpolated value with a difference in RLU of zero represented the best estimate of the number of target genome copies in the test sample (Fig. 4). The experimental values obtained in the different titration curves showed a linear correlation with expected values >100 target genome copies. To assess the repeatability of the chemiluminescent cPCR assay, we repeated the assay three times on the same day; the intraday CV was 1–10%.

To evaluate the diagnostic potentiality of our chemiluminescence cPCR technique, the chemiluminescence cPCR assay was used to quantify the viral DNA content in reference clinical samples. Six positive reference serum samples derived from patients in different phases of B19 infection, semiquantitatively titrated by endpoint dilution nested PCR (25), and six negative reference serum sam-

![Fig. 1. Kinetics of four different substrates for HRP: ECL (▲), ECL Plus (■), SS (▲), and SS-Ultra (○). Target sequence, 1000 B19 genome copies. Error bars, SD (n = 3).](image1)

![Fig. 2. Detection of different amounts of target DNA by chemiluminescent substrates: ECL (▲), ECL Plus (■), SS (▲), and SS-Ultra (○). Error bars, SD (n = 3).](image2)

![Fig. 3. Comparison between chemiluminescent (■) and chromogenic (○) detection of target DNA.](image3)
amplification to be achieved both when target and competitor sequences were amplified separately and when they were coamplified (5). The chemiluminescent immunoenzymatic reaction used to detect the different hybridized amplicons was performed in black microplate wells to achieve quantitative objective and specific results.

Different chemiluminescent substrates for HRP (ECL Plus, ECL, SS, and SS-Ultra) were analyzed for our assay. In fact, HRP chemiluminescent detection systems can use either the luminol-based enhanced chemiluminescence detection system (ECL, SS, and SS-Ultra) or a chemiluminescent process involving the enzymatic generation of an intermediate acridinium ester (ECL Plus). Among the four substrates used, ECL Plus gave more precise and sensitive results compared with the other compounds; this is in accordance with previous data showing that substrates generating acridinium esters have very good performance in analytical methods involving DNA detection (15).

In single PCR determinations, the detection limit with our chemiluminescent assay using ECL Plus was of 20 copies of both target and competitor sequences, which was lower than the detection limit for the colorimetric assay. The detection limit obtained with chemiluminescent detection reached the sensitivity of a nested PCR but avoided the possible risk of contamination that can occur in nested PCR assays (25).

In competitive titration curves in which target and competitor sequences were coamplified, titration values were reproducible with a linear correlation above 100 genome copies. This sensitivity of chemiluminescence cPCR seems appropriate for detecting viral infections occurring with very low viral titers in blood, for monitoring the clearance of a virus after acute infection, and for evaluating the potential risks of transmission of parvovirus through infected blood (10).

In our study, we also explored the potential of our chemiluminescence cPCR for diagnostic purposes in selected clinical samples because microplate luminometers similar to the one used in this work are beginning to be adopted in many diagnostic laboratories.

In the analysis of positive and negative reference serum samples, chemiluminescence cPCR was a specific and reliable assay for quantifying parvovirus DNA. In positive samples taken from subjects in different phases of B19 infection (early convalescent phase and chronic infection), the quantity of viral DNA measured with our assay correlated with the endpoint dilution positive in nested PCR assay and corresponded to the expected range of viral load in these clinical situations (23, 24); in negative samples, no viral DNA was quantifiable.

In conclusion, the assay described here, which uses a microplate luminometer, represents a highly sensitive, specific method for the quantitative detection of viral DNAs and is suitable both for research and diagnostic laboratories.

---

Table 1. Titration values obtained in positive reference serum samples by chemiluminescence cPCR.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Endpoint dilution positive in nested PCR</th>
<th>Chemiluminescence cPCR titration value, genome copies/μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$10^{-4}$</td>
<td>58 193</td>
</tr>
<tr>
<td>2</td>
<td>$10^{-3}$</td>
<td>17 725</td>
</tr>
<tr>
<td>3</td>
<td>$10^{-3}$</td>
<td>19 802</td>
</tr>
<tr>
<td>4</td>
<td>$10^{-3}$</td>
<td>4200</td>
</tr>
<tr>
<td>5</td>
<td>$10^{-2}$</td>
<td>1468</td>
</tr>
<tr>
<td>6</td>
<td>$10^{-1}$</td>
<td>277</td>
</tr>
</tbody>
</table>
This work was supported by CNR Target Project on “Biotechnology”, MURST (Ministero della Università e della Ricerca Scientifica e Tecnologica) and University of Bologna Funds for Selected Research Topics.

References