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Quantification of human cytomegalovirus DNA using the polymerase chain reaction

Jayne C. Fox, Paul D. Griffiths and Vincent C. Emery*

University Division of Communicable Diseases, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.

The important goal of developing quantitative assays for viral nucleic acids in clinical samples has been achieved for human cytomegalovirus (HCMV) by using a modified polymerase chain reaction (PCR). A control PCR target sequence was constructed by PCR mutagenesis to allow the post-amplification quantification of HCMV DNA. The control region was identical to a naturally occurring sequence within the glycoprotein B (gB) coding part of the virus genome, except that a unique restriction site, introduced by the aforementioned mutagenesis step, allowed post-amplification differentiation of control/non-control target amplified product. This technique was initially validated using known amounts of cloned control/non-control target DNA, and was found to be sufficiently sensitive to allow the quantification of a range of 10 to 10^6 genome equivalents of virus. The method was applied to urine samples of congenitally infected infants for which infectious virus titres were available. The results obtained demonstrated that the number of infectious virions determined by conventional cell culture represented a small proportion of the HCMV genome present in the samples, as assessed by the quantitative PCR methodology.

Since the initial description of the polymerase chain reaction (PCR) by Saiki and co-workers in 1988, the PCR has been increasingly applied to the detection of a variety of infectious agents within clinical material. The PCR can be utilized to detect both DNA and, following a reverse transcription step, RNA target sequences. Although replica PCRs on endpoint diluted samples (Simmonds *et al.*, 1990) does allow the quantitative detection of viral sequences, the majority of PCR applications are qualitative, i.e. the presence of the target sequence in the sample of interest will yield an amplified DNA fragment and hence a positive result. However, in many circumstances there are diagnostic and prognostic implications of being able to quantify the amount of virus present in a particular clinical specimen. For example, the prognosis of neonates congenitally infected with human cytomegalovirus (HCMV) is directly related to the amount of HCMV present in their urine at the time of presentation (Stagno *et al.*, 1986). Likewise, in patients infected with human immunodeficiency virus (HIV), progression to AIDS is accompanied by an increase in plasma virus load (Clark *et al.*, 1991). In such situations, conventional cell culture and $TCID_{50}$ determinations are of great benefit and cannot be replaced by existing qualitative PCR technology.

In the PCR system, amplification of target DNA initially occurs in an exponential fashion but ultimately the efficiency of amplification decreases owing to a number of factors, including limiting reagents and inefficiency of primer binding in the later cycles. Several methodologies have been investigated in an attempt to perform quantitative PCR, including the use of a co-amplified sequence, e.g. β-globin (Pang *et al.*, 1990), that is amplified simultaneously with the target sequence. Solution hybridization following PCR has also been investigated (Kellog *et al.*, 1990), as has the use of limiting dilution of the sample followed by PCR (Simmonds *et al.*, 1990). The latter method is accurate but time-consuming, expensive to perform and is unlikely to be suitable for the analysis of many samples, as would be required in a diagnostic laboratory or in studies involving large numbers of clinical specimens. The methods which use control primers that amplify other gene products or which use known amounts of target DNA in concurrent PCRs offer only a semi-quantitative PCR methodology since they cannot compensate for local variation in PCRs or the possible presence of inhibitory substances within specific samples. In addition, such methods do not compensate for the efficiency of the reverse transcriptase step in the amplification of RNA target sequences. Ideally, a quantitative PCR should compensate for all these variable (i.e. primer binding efficiency, inhibitory substances in the samples and variations between sample

wells in the thermal cycling device). Given the possible difficulties of quantitative PCR, we have chosen a different approach to meet the objective.

Ideally, any control sequence used for quantification should resemble the actual target sequence as closely as possible: it should possess identical primer binding sequences, G plus C content and number of bp between primer binding sites. Also, the control sequence should be added to each PCR mixture in order to compensate for sample-to-sample and sample–well variations. For such a system to function, a methodology to differentiate target from control amplimer is required. This requirement can be easily achieved by the introduction of a restriction enzyme site within the control amplimer via mutagenesis. Thus, for any given PCR, a pre-determined amount of control sequence is included in each reaction volume, the PCRs are performed and the amplimers are digested with the appropriate enzyme. The control sequence amplimer containing the restriction site is digested but the target sequence amplimer remains uncleaved. Subsequently, the products can be resolved by virtue of their size difference and finally the intensity of the two products is compared. Since a known amount of the control sequence has been amplified, within a given PCR the number of target molecules within the original sample can be back-calculated.

We have produced a control sequence to allow the quantification of an HCMV-specific PCR system currently in use in our laboratory (Darlington *et al.*, 1991; Fox *et al.*, 1991). A 149 bp sequence within the coding region of the glycoprotein B (gB) of HCMV was the target sequence for PCR amplification and thus served as the basis for the generation of the control DNA sequence. The construction of the control sequence is shown in Fig. 1. In essence, the difference between the control sequence and the authentic HCMV gB target sequence is a 2 bp change at bases 77 and 78 of the 149 bp amplimer, converting the original GG to a TT, as is shown in Fig. 1. These changes resulted in the introduction of a unique restriction site for *Hpa*I into the 149 bp target sequence. The mutagenesis was effected using the PCR as follows. Two complementary 24 bp oligonucleotides containing the altered nucleotide sequence (gB3 and gB4) were used in two separate PCRs: (i) using primers gB1 and gB3 and (ii) using primers gB2 and gB4 (see Fig. 1). The resultant 90 bp products of these PCRs were purified by standard methodologies (Sambrook *et al.*, 1989), mixed, heated to 95 °C for 10 min, then cooled to room temperature over a 30 min period. The resulting annealed products were 3′-extended using Klenow polymerase and deoxynucleoside triphosphates. Subsequently, a aliquot (1 μl) from this restriction mixture was used as the target for a PCR using 5′-phosphorylated primers gB1 and gB2 (Darlington *et al.*, 1991). The 149 bp product was purified, cloned into pUC13 and the mutation was confirmed by restriction endonuclease mapping and plasmid sequencing (Murphy & Kavanagh, 1988).

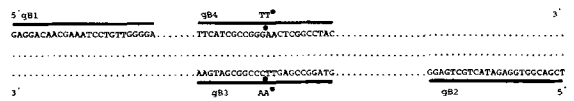

Fig. 1. Schematic representation of the 149 bp HCMV gB PCR target sequence. Primer binding sites and base sequences are shown for the amplification of the whole sequence (gB1 and gB2), and for the construction of the mutated control sequence. Base changes carried by gB4 and gB3 to produce *Hpa*I restriction sites are indicated by (●).

The potential of the control DNA sequence to facilitate quantitative PCR was assessed using the original 149 bp gB sequence cloned into pUC13 as the target DNA. Cloned target DNA ranging from 10^9 to 1 molecule was mixed with 10^3 molecules of the cloned control sequence and PCRs were performed, as described previously (Fox *et al.*, 1991) except that the primers gB1 and gB2 had been phosphorylated at their 5′ termini using [γ-^{32}P]ATP and polynucleotide kinase (Sambrook *et al.*, 1989) to allow subsequent detection of the amplified products by autoradiography. After amplification, the PCR products were phenol-extracted and precipitated with ethanol prior to digestion with *Hpa*I. The products were then separated on a 20% polyacrylamide gel and detected by autoradiography and scanning densitometry (model ACD 2000; Gelman Sciences). A typical densitometric scan is shown in Fig. 2. Since only the control sequence amplimer was susceptible to digestion with *Hpa*I, the faster migrating fragments represented the digestion products (77 and 72 bp, unresolved), whereas the slow migrating fragment represented the 149 bp uncut product of the target DNA amplimer. Such analyses were carried out for each PCR within the titration. A graphical representation of the data obtained from four separate titration experiments is shown in Fig. 3. These data show that when 1000 molecules of the control DNA are included in the PCR the system allows between 10 and 10^6 molecules of the target sequence to be quantified, corresponding to a dynamic range of 10^5. In each experiment a control for the efficiency of the restriction digest, i.e. a PCR with only control sequence as target, was also performed. However, it was possible that at least some of the control amplified product was not susceptible to digestion, due to the annealing of single strands of control and target amplified sequence. These products would be expected to make up a considerable proportion of the PCR product, but only if 100% of all amplified product denatured completely at the 95 °C step of the PCR. This

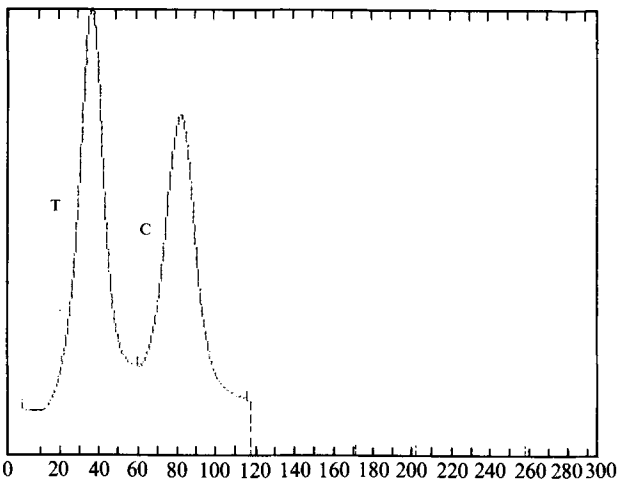

Fig. 2. Densitometric scan of digested PCR products following the co-amplification of 10^3 copies of control sequence (C) and 10^3 copies of non-mutated target DNA (T). The proportion of signal due to each species was 52·5% for T and 47·5% for C. The migration of the products from the origin is shown in millimetres.

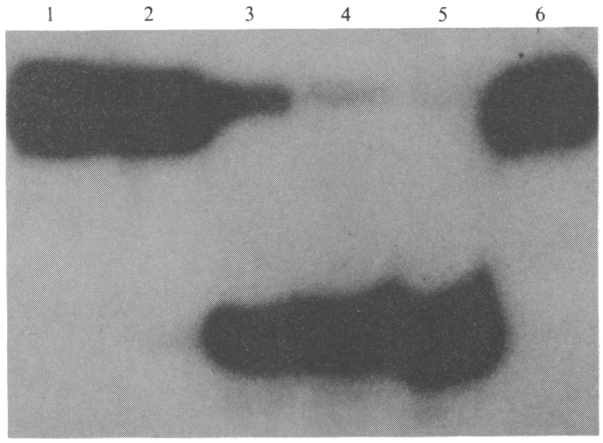

Fig. 4. Autoradiograph of PCR-amplified, *Hpa*I-digested and electrophoretically separated PCR products. Each of lanes 1 to 5 shows the results of DNA amplification of 10^3 copies of control sequence, together with target DNA purified from the urine (sample A) of a congenitally infected infant. $TCID_{50}$ values were used to estimate the number of infective genomes amplified in each individual reaction. These values were 500, 50, 5, 0·5 and 0·05 for lanes 1 to 5, respectively. Lane 6 shows an undigested control electrophoresed concurrently as a molecular size marker.

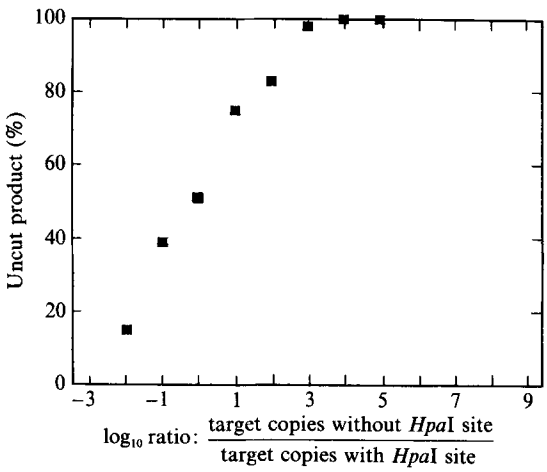

Fig. 3. Relationship between the percentage of PCR amplified product remaining uncut, after digestion with *Hpa*I, and the copy ratio of target sequence to control sequence amplified (expressed as $\log_{10}$). Each point represents the mean of four sets of data obtained from four individual experiments.

is unlikely (Becker-Andre & Hahlbrock, 1989) and the true situation is probably somewhere in between, i.e. heterologous sequences are found but not in large numbers or in a predictable way. However, from the data shown in Fig. 3 the phenomenon may only be apparent at high concentrations of target DNA.

The utility of the quantitative PCR system described above to provide important information of clinical relevance was assessed by investigating the amount of HCMV in urine samples from two neonates congenitally infected with HCMV. It has been shown previously that the prognosis of such infants is directly related to the viral load in the urine (Stagno *et al.*, 1986). The two urine samples (A and B) were initially analysed by conventional cell culture $TCID_{50}$ determinations (Grist *et al.*, 1966) and shown to contain approximately $2{\cdot}5 \times 10^2$ and 5×10^2 infectious units of HCMV/ml urine, respectively. The same urine samples were processed by the methods described by Kimpton *et al.* (1991) to extract DNA, and dilutions based on the $TCID_{50}$ results were used in the quantitative PCR analysis. The autoradiography results obtained from a typical quantitative PCR analysis of urine A are shown in Fig. 4. The quantitative PCR system showed that the urine samples A and B contained approximately 5×10^5 and 10^6 genome equivalents/ml urine, respectively. These results imply that clinical samples can yield quantitative data, and that within these specific urine samples there are approximately 10^3-fold more genomes than is estimated from $TCID_{50}$ determinations and therefore a considerable quantity of virus present in these samples is either not infectious in the assay system used (primary human embryo lung fibroblasts), or the viral nucleic acid is not replication-competent. Our data reinforce those obtained by previous studies in which significantly more viral genomes were present than estimated by $TCID_{50}$ determinations. For example, Benyesh-Melnick *et al.* (1966) demonstrated that the number of CMV particles/p.f.u. for two laboratory strains of CMV (AD169 and C-87) ranged from 160 to 1050. Fig. 4 also clearly

demonstrates that the autoradiographic image, which reflects the digested PCR amplified products, is not straightforward to interpret. At high copy numbers of sample-derived target the relationship between cut and uncut amplified product is complex, possibly due to the presence of heterodimers. This can easily be overcome, however, by sample dilution and repeating the PCR at lower sample-derived copy numbers.

In conclusion, we have used a relatively rapid, simple methodology whereby an HCMV PCR system can be quantified by using a sequence which mimics the target sequence in all respects except for the possession of a unique restriction endonuclease site. The dynamic range allows between 10 and 10^6 molecules of target nucleic acid to be accurately quantified when 1000 molecules of the control sequence are included in the PCR. Such a dynamic range exceeds that of the post-PCR quantitative method described by Henco & Heibey (1990). The approach truly compensates for any variation in individual PCRs since the control sequence is amplified in the same environment as the target sequence. The utility of the system to provide important clinical data has been shown using urine from neonates congenitally infected with HCMV. Currently, $TCID_{50}$ determinations take at least 3 weeks to perform, whereas the quantitative PCR can be effected in less than 2 days. It is important to determine whether the quantification of viral DNA by this method can provide meaningful clinical data. Indeed, preliminary results obtained from renal transplant patients, whose low titres of HCMV in the clinical sample under investigation preclude $TCID_{50}$ determinations, suggest that quantification by this method may provide prognostic information (data not shown). The use of alternative non-radioactive methods, e.g. fluoresceinated primers and scanning laser densitometry to detect the products, is also possible. Since the control sequence can be cloned into riboprobe-type vectors, it will be possible to produce an RNA copy of the control sequence for quantification of infectious agents containing RNA genomes (for example, Steiger *et al.*, 1991). Such systems will prove invaluable in investigations into the effects of antiviral agents on plasma levels of HIV in infected individuals. All these applications and modifications are currently being pursued in our laboratory.

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