

Development of a Multiplex PCR for the simultaneous amplification and genotyping of glycoprotein N among human cytomegalovirus strains

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SUMMARY

Genomic variation among human cytomegalovirus (HCMV) strains is probably involved in HCMV-induced pathogenesis. The envelope glycoprotein N (gN) showed extensive genetic polymorphism as HCMV isolates have been clustered into four distinct gN variants (gN-1, gN-2, gN-3, gN-4) whose distribution has been analyzed worldwide using different methodological approaches (PCR-RFLP, PCR-Cloning, PCR-Sequencing).

This paper describes a new method for concurrent detection of gN genotypes among HCMV strains using a multiplex gN-variants specific PCR plus visualization on agarose gel, avoiding subsequent steps such as cloning, restriction or sequencing. This novel approach will reduce costs and shorten the detection time of gN polymorphisms among HCMV clinical isolates.

KEY WORDS: Human cytomegalovirus, Gene polymorphisms, Wild-type strains, Glycoprotein N, Multiplex PCR

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Human cytomegalovirus (HCMV) is a beta-herpesvirus causing severe diseases displaying a wide spectrum of clinical manifestations, particularly in immunocompromised individuals or congenitally infected newborns (Ho, 1990; Gaytant *et al.*, 2002).

The mechanisms responsible for the severity and duration of clinical manifestations remain unsettled, but host factors together with viral determinants are thought to play a decisive role.

Research into the infecting strain has focused on genetic variability among HCMV wild-type isolates (Meyer-Konig *et al.*, 1998; Shepp *et al.*, 1998; Arav-Boger *et al.*, 2006). Although several studies

suggest that different strains of cytomegalovirus are 95% homologous, polymorphic ORFs have been detected in coding and non coding regions of the virus genome. Most of the variable regions are strongly clustered in a few dominant genomic variants defined as "genotypes" (for review, see Pignatelli *et al.*, 2004).

Among these variable regions, HCMV ORF UL73 turned out to be one of the most polymorphic genes among HCMV clinical strains (Dal Monte *et al.*, 2004). This gene encodes the immunogen envelope glycoprotein N (gN), a gC-II component implicated in virus attachment to the host cell and spread (Kari and Gertz, 1993; Mach *et al.*, 2000; Dal Monte *et al.*, 2001; Pignatelli *et al.* 2001; Burkhardt *et al.*, 2009).

The 5' sequence of the ORF UL73 showed a polymorphic region involving the N-terminal domain of gN and clustered in four main gN genomic variants: gN-1, gN-2, gN-3 (with subgroups gN-3a, gN-3b) and gN-4 (with subgroups gN-4a, gN-4b, gN-4c) (Pignatelli *et al.*, 2003a; Pignatelli *et al.*, 2004; Dal Monte *et al.*, 2004). gN variants are

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linked to HCMV gO polymorphisms and seem to be implicated in HCMV pathogenesis (Pignatelli *et al.*, 2003b; Mattick *et al.*, 2004; Rossini *et al.*, 2005; Pignatelli *et al.*, 2006; Lanari *et al.*, 2008; Yan *et al.*, 2008; Pignatelli *et al.*, 2010).

Several studies have documented UL73-gN hypervariability using time-consuming and expensive approaches, such as PCR-sequencing, PCR-RFLP or PCR-cloning to amplify and genotype UL73 from the HCMV genome (Pignatelli *et al.*, 2003a; Rossini *et al.*, 2005; Beyari *et al.*, 2005; Puchhammer-Stöckl *et al.*, 2006; Novak *et al.*, 2008; Chen *et al.*, 2008; for comparative overview, see Pignatelli and Dal Monte, 2009).

This study aimed to develop a new method for concurrent detection of gN genotypes among HCMV strains using a multiplex gN-variants specific PCR, avoiding subsequent steps such as cloning, restriction or sequencing. This novel approach will improve the detection of gN polymorphisms from HCMV clinical isolates in terms of costs and time.

Multiplex PCR can amplify more than one target sequence including more than one pair of primers in the reaction. This approach has the potential to produce considerable savings of time and effort, without compromising test utility and has been applied in the field of infectious diseases, mainly to detect multiple pathogens in a single sample for diagnostic purposes (Elnifro *et al.*, 2000).

Based on the many reviews describing the key parameters influencing the performance of both uniplex and multiplex PCRs, and supported by dedicated software for priming analyses, we designed a new set of primers which should anneal selectively with specific regions of each gN genotype, minimizing the formation of primer dimers or non-specific interactions, and improving sensitivity at the specific target. Due to PCR drift and selection, especially during the first amplification cycles, our multiplex method was optimized by empirical testing and a trial-and-error approach, because there is no way to predict the real performance of a selected primer set even if it meets the general parameters of primer design.

ORF UL73 is 417 nt long, but its polymorphic region comprises only about the first 261 nt, corresponding to the first 87 aminoacids of the gN glycoprotein. Thus, to amplify the four main gN genotypes (gN-1, gN-2, gN-3, gN-4) selectively in

a single step and distinguish them simply on the basis of the relative length of the amplification products, we used a single gN-common lower primer and distinct gN specific upper primers spanning different regions of the polymorphic ORF.

Upper primers were selected studying the alignment of prototype strains and 42 HCMV clinical isolates collected worldwide (six sequences for each gN genotype, representative of the intragenotype variability), obtained using the MegAlign software (DNA Star, Lasergene 7.0). Due to the extreme difficulty in selectively amplifying all seven gN sub-groups simply based on differences in amplicon length, the intragroup variability was also taken into consideration and we chose to distinguish gN-1, gN-2, gN-3b, gN-4a subgroups. gN-4b and 4c (99-100% intersubgroup variability) were both concurrently amplifiable by the same upper primer. The gN-3a variant was gathered by the absence of amplification product due to the lack of the gN-3a specific upper primer in the multiplex gN-PCR mix. This case should require a further confirmation of the gN-3a genotype by PCR-sequencing.

Figure 1 reports the alignment of HCMV strains distinguished on the basis of their gN genotype and the relative position of specific primers for a selected genotype.

Following the previous step, Oligo 5.0 software (Med Probe, Oslo, Norway) for primer analysis was employed to verify the effectiveness of the selected primers, the reliability of the PCR reaction, the suggested annealing temperature and primer compatibility.

As far as the PCR conditions are concerned (MgCl₂ and primers concentration, annealing temperature, number and duration of cycles), gN-multiplex PCR was optimized on clinical strains with already known genotypes representing the entire set of the seven gN genomic variants. A single step PCR was performed using a single lower primer (multigN-lw: 5'-GACATTGCTGCTTCCAGAA-3') and a set of five upper primers (gN-1: 5'-TTCTGCTAGCGTATCAACTACC-3'; gN-2: 5'-AGT-GCAAAACACTGGTGCT-3'; gN-3b: 5'-CACAAACACATTAACGAGT-3'; gN-4a: 5'-CAACAAT-ACGTCGACTGCTAGCACAC-3'; gN-4b/c: 5'-GACAACACTAGTACAACACTACGGTGACAA-3'), displaying a specific annealing region on ORF UL73, typical of a single gN variant. For the multiplex

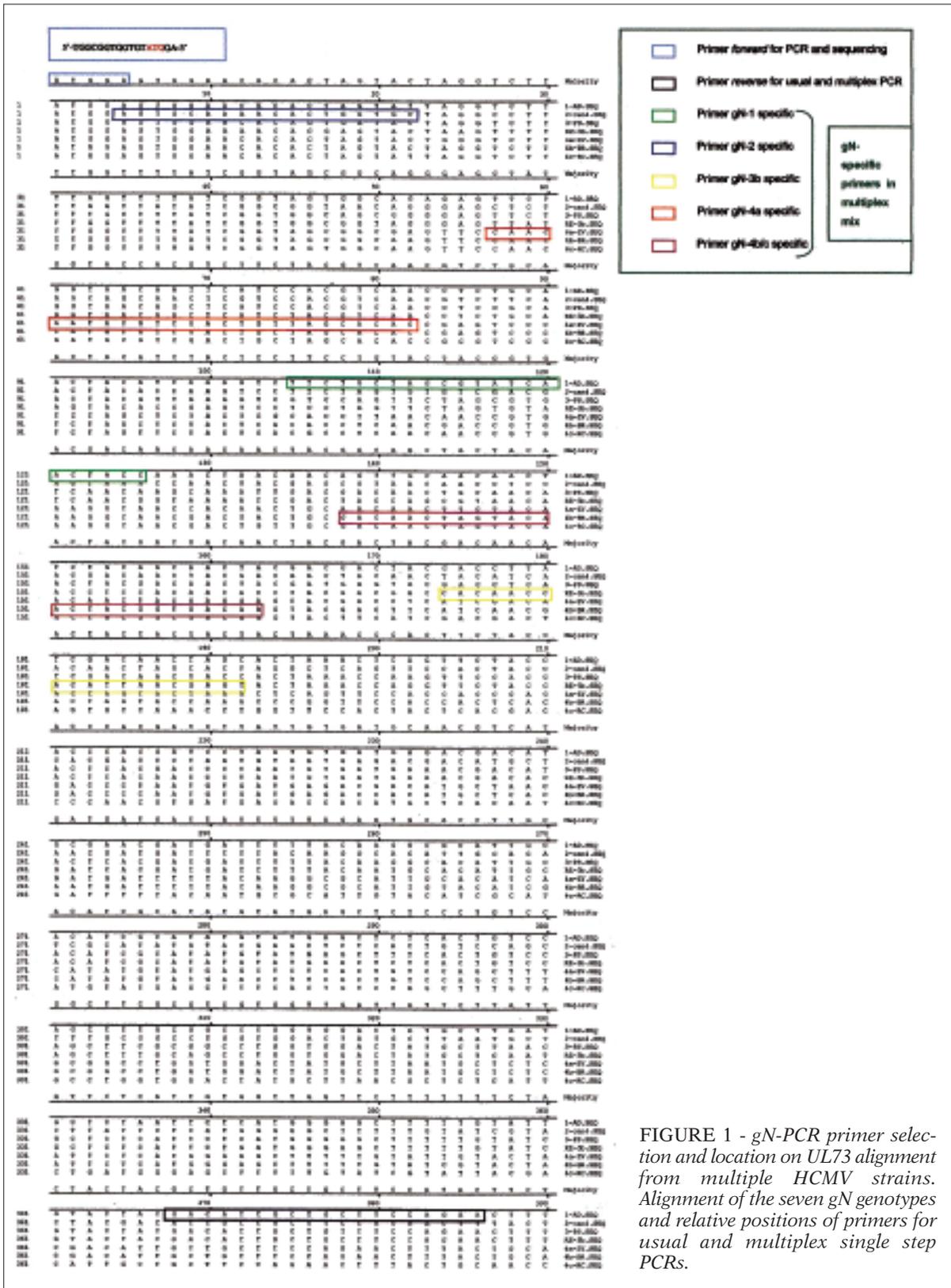


FIGURE 1 - gN-PCR primer selection and location on UL73 alignment from multiple HCMV strains. Alignment of the seven gN genotypes and relative positions of primers for usual and multiplex single step PCRs.

TABLE 1 - Primer sequences and expected amplicon length in multiplex gN amplification.

<i>gN</i> -type specific upper primers*	Product length
gN-1: 5'-TTCTGCTAGCGTATCAACTACC-3'	283 bp
gN-2: 5'-AGTGCAAACACTGGTGCT-3'	380 bp
gN-3b: 5'-CACAACCACATTAACGAGT-3'	214 bp
gN4a: 5'-CAACAATACGTCGACTGCTAGCACAC-3'	325 bp
gN-4b/c: 5'-GACAAC TAGTACAAC TACGGTGACAA-3'	244 bp

Common multigN-lower primer: multigN-lw: 5'-GACATTGCTGCTCCAGAA-3'

*Upper primers optimal annealing temperature ranges between 51,2°C and 56,1°C. gN-3a variant is identifiable by the absence of amplicon due to the lack of its specific upper primer in the multiplex-gN mix.

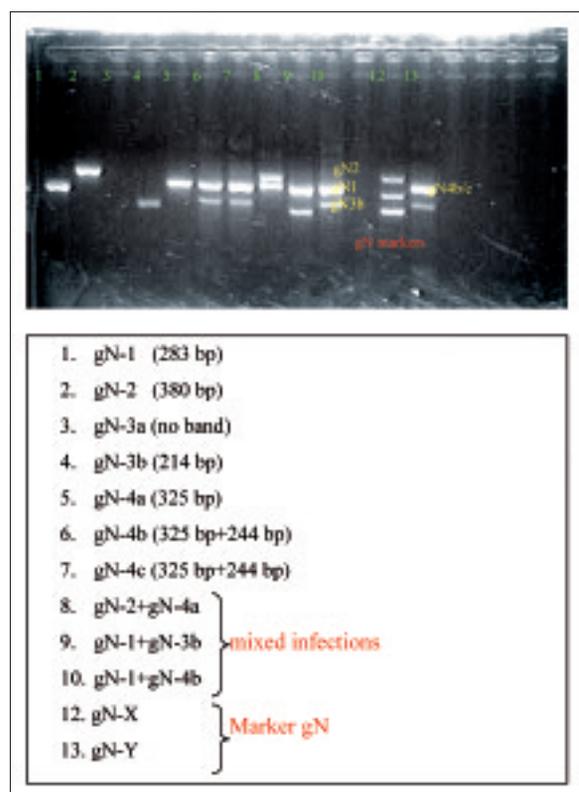


FIGURE 2 - Electrophoretic pattern of gN variants amplified by multiplex PCR from the HCMV genome of clinical isolates. Typical 3.5% agarose gel showing the electrophoretic pattern expected for each gN variant tested by multiplex single step PCR. "Artificial" HCMV mixed infections (gN-2+gN-4a; gN-1+gN-3b; gN-1+gN-4b) built up using viral DNAs already genotyped at the UL73-gN locus are efficiently detectable by multiplex gN amplification. gN-X and gN-Y: home-made markers for gN variants.

PCR reaction an equimolar mix of the selected primers was used. For the amplification reaction, a 50 μ l reaction was set up with 200 μ M dNTPs, 0.2 μ M of each primer, 1X PCR Buffer II (Perkin-Elmer, Branchburg, NJ-USA), MgCl₂ 1,5 mM and 2 unit of AmpliTaq Gold[®] (Perkin-Elmer, Branchburg, NJ-USA) DNA polymerase. Amplification was carried out with a PTC-200 Thermal Cycler (MJ Research Inc., Watertown, MA-USA) on 300 ng of total DNA under the following conditions: after a hot start step for AmpliTaq Gold[®] activation, samples underwent 35 cycles of denaturation at 96°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min.

Negative (mix without DNA) and positive (mix with viral DNA of known gN variant) controls were added in each round of amplification. Multiplex products were analyzed by electrophoresis on 3.5% agarose gels stained with Gel Red[™] (Biotium, Hayward, CA-USA), where each gN type specific amplification product shows an unequivocal length.

Table 1 summarizes multiplex-PCR amplification conditions, the set of primers and the length of the PCR products expected for each specific genotype.

On basis of the electrophoretic pattern detected, each isolate tested was assigned to one out of the seven gN genotypes, using a combination of the appropriate molecular weight markers (50 bp ladder, M18 and M20, Mbi Fermentas, Hannover,

MD-USA). A multiplex-gN specific molecular weight marker was finally prepared mixing the appropriate amounts of the amplification products obtained from gN-prototype strains to be used concurrently with the usual molecular weight markers in the following tests on unknown specimens (gN-X and gN-Y in Figure 2). Figure 2 reports a typical 3.5% agarose gel showing the electrophoretic pattern expected for each gN variant tested by multiplex single step PCR. As shown in the figure, "artificial" HCMV mixed infections were built up using viral DNAs already genotyped at the UL73-gN *locus* and they are also efficiently detectable by multiplex gN amplification.

The new procedure was validated on 75 HCMV clinical isolates collected at the Microbiology Unit of S. Orsola General Hospital, Bologna, Italy from pregnant women with acute HCMV infection ascertained by serological tests, congenitally-infected newborns who were proved to have acute HCMV infection by virus isolation from urine (Pignatelli *et al.*, 2010) and mother's milk displaying a HCMV reactivation following lactation (Capretti *et al.*, 2009) documented by positive virus isolation (Gleaves *et al.*, 1984). Specimens were derived from amniotic fluid (12), newborns' urine (53 samples) or mothers' milk (10).

Sample collection, virus isolation from urine, amniotic fluid and milk, and viral DNA extraction by QIABlood kit with BioSprint15 (QIAGEN, Hilden, Germany) were performed as previously described (Lanari *et al.*, 2008; Pignatelli, *et al.*, 2009; Capretti *et al.*, 2009; Pignatelli *et al.*, 2010). gN genotyping was performed on the collected samples in parallel and blinded by PCR plus sequencing as control reaction and the new optimized single step multiplex PCR. HCMV DNA from gN prototype strains were used as positive control. Appropriate negative controls were used to check for contamination during amplification procedures. The single step PCR to amplify the entire UL73 ORF used the previously reported primer set (forward UL73: 5'-TGGCGGTGGTGTGATGGAG-3'; reverse UL73: 5'-GACATTGCTGCTTCCAGAA-3') and PCR conditions (Chen *et al.*, 2008; Pignatelli *et al.*, 2009). The concordance of PCR-sequencing and multiplex-PCR is 97.3% (73/75 samples). One discordant case was due to the presence of a mixed infection in a single sample of mother's milk (Lanari *et al.*, 2008), while in

the other case the amplification product was not interpretable, probably due to degradation of the original DNA sample.

In conclusion, this present work briefly describes a novel method to genotype UL73-gN variants among HCMV wild-type strains. This approach uses the multiplex-PCR technique and a simple 3.5% agarose gel visualization of UL73 PCR products, readily distinguishable by differences in length. This approach is cheaper and faster than other methods published to date (for review see Pignatelli and Dal Monte, 2009) and can easily be implemented by both basic research and routine diagnostic laboratories lacking facilities for sequencing or cloning procedures.

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