

HCMV gB GENOTYPES IN CERVICAL SECRETION AND PLACENTA TISSUES IN THE STATE OF ESPÍRITO SANTO, SOUTHEASTERN BRAZIL

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ABSTRACT

Human cytomegalovirus (HCMV) displays genetic variability in several regions, supposed to be related with strain-specific tissue tropism and immunopathogenesis. Based on sequence variation in the UL55 gene that encodes gB glycoprotein, HCMV strains can be assigned to one of four genotypes. Previous studies have addressed gB genotyping mostly by investigating strains derived from immunosuppressed patients, sometimes without previous knowledge about genotype distribution in a geographic area. The present study verified the distribution of HCMV gB genotypes of strains obtained from immunocompetent women at Vitória City, Espírito Santo State, Southeastern, Brazil. The HCMV genome was extracted from their cervical secretion, fetal and maternal placenta tissues (chorionic villous and decidua) from abortion cases and from white blood cells (WBCs). HCMV genotyping was performed by restriction fragment length polymorphism analyses of amplified product from the high variability site of the UL55 gene. All four genotypes were observed in both cervical secretion and placenta, whereas in WBCs a single gB1 genotype was detected. HCMV gB1 and gB2 genotypes were detected, respectively, in nine and in six of the 23 studied samples, while gB3 and gB4 were each found in four separate samples of the total. The differences in genotype frequency were not considered statistically significant. No mixed genotype infection was observed. The results indicated that the four gB HCMV genotypes had no particular tropism for placenta tissues and that all genotypes circulated within immunocompetent women at the time and in the region of study.

Key words: cytomegalovirus; RFLP; gB genotypes; cervical secretion; placenta tissues

INTRODUCTION

Human cytomegalovirus (HCMV), a widely distributed β -herpesvirus, is the leading agent of congenital infection (2,38), causes significant morbidity and mortality among immunocompromised patients (16,23) but is seldom responsible for symptomatic infections in immunocompetent patients (19). HCMV establishes latent infection associated with reactivation episodes during which it may be excreted from

saliva, urine, cervical secretion, sperm and breast milk (12,28,37).

HCMV strains display nucleotide polymorphism in several functionally important genes that encode gB (UL55), gH (UL75), gN (UL73) glycoproteins or UL144 region, supposed to be involved in viral virulence and explored as genotype systems. HCMV gB glycoprotein, the major antigen involved in induction of neutralizing antibodies (11), is associated with adsorption, penetration of the host cell, and cellular fusion

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(24,25,41); gH glycoprotein is related with cellular fusion (18); gN, with adsorption and cell to cell dissemination (31) and UL144 encodes for a putative member of the tumor necrosis factor- α -like receptor superfamily with unknown function (21,30).

Based on the restriction fragment length polymorphism (RFLP) of amplification products of HCMV gB (bases 1319-1604), four patterns (gB1-gB4) have been observed (9,10). According to these profiles, a genotyping system was then proposed and conventionally accepted as a putative marker of development of HCMV-associated disease.

In this direction, several studies have been carried out that have attempted to verify an association between the gB HCMV genotype and disease manifestations. Some have supported a possible association between viral tropism/pathogenicity and the gB gene (15,22) whereas others have found no evidence whatsoever of a gB-virulence association (17,33,42). But, to date, little attention has been given to HCMV strains in immunocompetent individuals (4,27) and by this time, concern has begun on those strains involved in congenital infection (4-6,20,29,39).

Studies of HCMV gB genotypes have been done directly from clinical specimens: blood, urine, semen and/or saliva (3,9,13,22,33,44), or after viral isolation in cell culture (4,5,27,39,42). At the present time, studies on HCMV gB genotypes employing such clinical specimens as cervical secretion and placenta tissues (chorionic villous or decidua) are absent or with rare description (40). Therefore, the aim of this study was to describe the frequency of HCMV gB genotypes from strains detected in cervical secretion from healthy women, and those that could infect placenta. HCMV strains were directly genotyped from clinical samples, with the advantage of avoiding mutation during viral *in vitro* replication while favoring the selection of strains affording greater opportunity for cellular replication (8). The revealed genotypes would permit future support on congenital infection and fetal or newborn tropism and consequences beyond the relevance on epidemiological knowledge.

MATERIAL AND METHODS

Specimens

Twenty three HCMV strains were derived from the following positive specimens: i) 12 cervical secretions obtained from 102 immunocompetent pregnant and non-pregnant women (mean age 26 years), ii) eight placenta tissues (deciduas or chorionic villi) obtained from 88 abortion cases, and iii) three obtained from white blood cells (WBCs) – two from 100 pregnant and non-pregnant women and one from 95 women undergoing an abortion (35,36). Each strain was recovered from a different woman. Sample handling, storage and DNA extractions have been previously reported (35,36).

PCR and nested PCR for gB gene

Extraction products were subjected to gB gene HCMV amplification corresponding to high variability in the gB gene sequence. The outer and inner gB primers (gB1868N, gB829N and gB1319N, gB1604, respectively) were used in accordance with the criteria previously described (33). The amplification protocol used was established in the present paper. PCR and nested-PCR mix solutions contained in a total volume of 50 μ L: 1.5 mM MgCl₂, PCR buffer (20 mM tris-HCl pH 8.4, 50 mM KCl), 200 μ M each of the four dNTPs, 1.5 units of Taq DNA polymerase (Life Technologies™, Inc., MD, USA) and 20 pmol of each outer or inner primers. For PCR reaction, 15 μ L of extracted DNA was added to 35 μ L of mix solution. The first round of amplification was undertaken with one denaturation cycle at 95°C for 5 min and thirty-five amplification cycles consisting of 60 sec each at 94°C and 90 sec for annealing and primer extension at 72°C, followed by a 72°C final extension for 5 min. The PCR products were used as a template in a nested-PCR assay. The second round amplification was undertaken with initial denaturation at 94°C for 2 min, followed by thirty-five cycles consisting of 30 sec each at 94°C and 30 sec at 72°C and then by a 72°C final extension for 5 min. All precautions to avoid cross-contamination and amplification inhibition were observed by using water and β -globin gene amplification as negative and positive controls, respectively, during all procedures (35,36). PCR and nested-PCR resulted in DNA fragments of approximately 1036 bp and 305 bp, respectively, which were resolved on a 1.5% agarose gel (Invitrogen™, Carlsbad, CA, USA) and stained with 0.5 μ g/mL ethidium bromide (Sigma, St Louis, MO, USA).

Restriction Fragment Length Polymorphism Analysis

To identify the HCMV genotype, nested-PCR products were separately digested with 10 units of restriction endonucleases *RsaI* and *HinI* (Invitrogen™, Inc., MD, USA), after incubation in the appropriate buffer at 37°C overnight. The digestion products were resolved on 4.0 % MetaPhor agarose gels (FMC BioProducts, Rockland, Maine, USA) and stained with ethidium bromide (Sigma).

RESULTS

The gB digestion products were adequately assigned to one of the gB1-gB4 genotypes (Fig. 1) according to the restriction fragments (estimated to be from 36 to 240 bp) as previously proposed (9). All four HCMV gB genotypes were found in the 12 vaginal secretions collected from both immunocompetent pregnant and non-pregnant women as well as from the eight abortion tissues (Table 1). No particular genotype was found to predominate in either of these two groups and no mixed infection was observed. The HCMV gB1 genotype was the only one recovered in the three WBCs

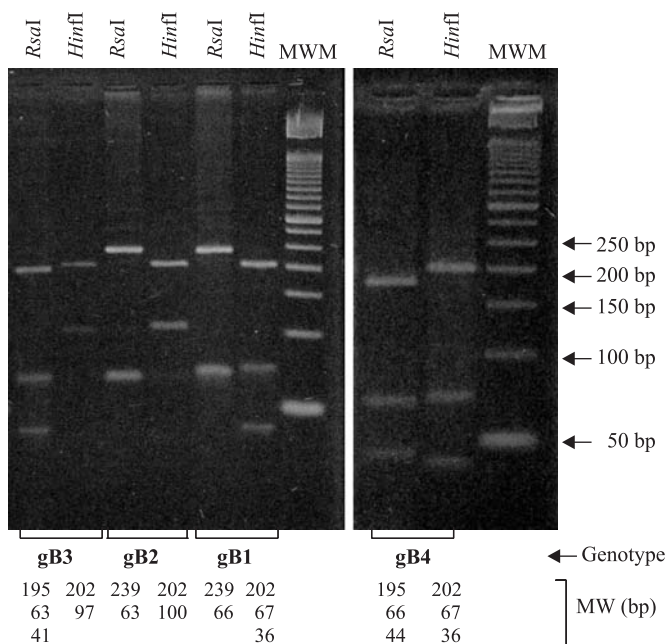


Figure 1. Ethidium-bromide-stained 4% MetaPhor agarose gel, showing patterns of amplified *gB* gene (1319-1604 bases) fragments following restriction digestion. *RsaI* and *Hinfl* enzymes yielded distinguishable digest profiles for groups 1-4 as defined in the figure. *gB* genotypes and estimated weights of digestion fragments [9] are below each lane. MWM: 50 bp molecular weight marker. MW: estimated molecular weights.

Table 1. HCMV *gB* genotype distribution among clinical specimens obtained from immunocompetent women.

HCMV <i>gB</i> Genotype	Clinical Specimens			
	Secretion ^a	Tissue ^b	WBCs ^c	Total ^d
gB1	4	2	3	9 (39.1 %)
gB2	3	3	0	6 (26.1 %)
gB3	2	2	0	4 (17.4 %)
gB4	3	1	0	4 (17.4 %)
Total	12	8	3	23

^aCollected from pregnant and non-pregnant women;

^bCollected from abortion cases;

^cWBCs = white blood cells, collected from pregnant (1), non-pregnant (1) and abortion cases (1);

^dStatistical analysis was performed using the χ^2 test ($\chi^2=2.91$; $P=0.40$).

specimens. *gB1* genotype seemed to predominate in all the investigated samples (9 out of 23), followed by *gB2* (6 out of 23) and by *gB3* (4 out of 23) and *gB4* (also 4 out of 23).

Although, percentage-wise, differences were observed, they were not found to be statistically significant ($P=0.40$), as analyzed by EPI-Info version 6.04b using chi-square test.

DISCUSSION

Genotype characterization of HCMV strains that circulate in a population in a certain geographic area is of utmost importance to elucidate studies on viral tropism and virulence markers. In this study we identified the four *gB* genotypes, previously described (9,10), from immunocompetent women, in both cervical secretion and placenta.

It is noteworthy that, although limited by the small number of HCMV strains from tissues obtained from the 88 abortions cases, *gB1-gB4* genotypes could be observed on chorionic villi and/or deciduas. HCMV found in placenta tissues from abortion cases can represent latent and/or active viral infection. The virus could conceivably reach the placenta tissue by hematogenic dissemination or contiguous endometrial cell infection (14,34). Therefore, taking into account that the four HCMV *gB* genotypes were also detected in vaginal secretion, it would be reasonable to expect that abortion tissues could be infected with any one of these strains. The fact that the four HCMV *gB* types were also found in placenta tissues suggests that there is no specific genotype tropism for placenta tissue infection. However, whether the strains recovered on placenta tissue would reflect the strains in congenital infection remains to be determined.

As recently reported, all four *gB* genotypes were capable of causing congenital infection in North American, Italian and French babies (4-6,29) and in Chinese infants, except by *gB4* genotype (43). Similar findings were observed when *gN* or *UL144* were used as virulence markers (29,32). Any link between the genotypes and the severity of congenital diseases were associated with specific genotypes in the previous mentioned studies, although Arista *et al.* (4) suggested that *gB1* could have a peculiar tropism for placental tissues. In contrast with these above citations, Lukácsi *et al.* (20), in Southern Hungary, found only the *gB1* genotype in congenital infection, but the genotypic distribution remained to be elucidated in that population. This fact highlights the importance of our study on establishment of genotypes distribution in specimens more probable to be responsible for congenital infection. Interesting report on *gB* distribution in Australian subjects was that certain *gB* genotypes were associated more commonly with childhood infections and that these differed from strains associated with invasive disease in HIV adult patients (39).

Distinct HCMV strains had infected the mother after her first pregnancy undergoing a second gestation whose newborns had symptomatic HCMV congenital infection (7). Actually, HCMV remains a problem, calling for immediate additional research in order to elucidate the real potential risks for a

newborn in regions with high HCMV seroprevalence rates like Brazil, where multiple genotypes circulate, as demonstrated in the present study. Despite the disappointing reports on gB or UL144 genotypes as a prognostic value in HCMV congenital infection (4-6,29,30), prospective studies indicate that gB genotypes could serve as a marker of HCMV maternal reinfection by a distinct strain leading to placental and fetal infection.

It is worth pointing out that HCMV seroprevalence in Brazil is around 45% in children from four-to-nine months of age and as high as 98% among adults (26,36). The sexual transmission of HCMV has been well established (12), which expose to the continuous viral reinfection and to the occurrence of distinct strains coinfecting the same individual. Nevertheless, in the present study, no mixed HCMV gB genotype infection could be found, which in no way means that the women under study were infected with only one strain. It could reflect that, among immunocompetent women, only one particular strain at a time is reactivated or even that one particular strain may have been detected in a latent state to the detriment of another. Interestingly, the results of this study are in juxtaposition to those of Aquino and Figueiredo (3), who reported a high percentage of HCMV coinfection simultaneously detected in the same or distinct clinical specimens, or in sequential samples, obtained from bone marrow transplant recipients in Ribeirão Preto, São Paulo, in Southeastern Brazil. The population samples of the two studies are quite distinct so that immunosuppression could have favored the viral reactivation of more than one HCMV gB genotype either at the same or within a very short period of time. Therefore, to better evaluate the possibility of coinfection occurring among immunocompetent women, it would be interesting to follow the course of viral excretion over a prolonged length of time.

While all four above-cited HCMV genotypes were observed in both vaginal secretion and abortion tissue samples, only genotype gB1 was detected in their three WBCs samples. Arista *et al.* (4) found a predominant circulation of gB3 and gB4 in immunocompetent Italian subjects while Ahumada-Ruiz *et al.* (1) related gB1 and gB2 in pregnant women and gB2 in blood donors from Costa Rica. Therefore, a different geographic distribution of HCMV gB genotypes is evident from these studies. To determine which genotype is more closely associated to hematogenic dissemination in an immunocompetent population, it would be necessary to study a much larger number of WBC samples and then attempt to correlate the various HCMV types, such as the ones found in others clinical samples as leukocytes, saliva, urine as well as semen.

The results of this study might be of interest to determine the predominance of a particular HCMV gB genotype in congenital infection, regardless the stage in the process. The answer to that question would probably provide some valuable additional information on the possibility of an association between the gB genotype and virulence.

Up to now, a number of reports have indicated that the restriction analyses of gB gene products remain uncertain as a reliable virulence marker, at least as a single marker. However, they are important in epidemiological studies and should be compared to available data on other putative genetic markers of virulence. Furthermore, as the target of neutralizing antibodies, it would also be interesting to investigate whether gB might be a marker of coinfection frequency.

Finally, even though a rather limited number of clinical specimens were studied, this report clearly demonstrated that the four different HCMV gB genotypes described herein circulated widely in Southeastern Brazil. However, the genotype distribution amongst other different clinical specimens, as saliva or semen, has yet to be determined. Furthermore, an evaluation of the geographic and demographic differences in the frequency of gB genotypes would be useful to determine the role of gB genotypes in virulence.

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RESUMO

Genótipos gB de citomegalovírus humano em secreção cervical e placenta no Espírito Santo, Sudeste do Brasil

O citomegalovírus humano (HCMV) apresenta variabilidade em diversas regiões do genoma, supostamente relacionada ao tropismo tecidual e imunopatogênese viral. Baseando-se na variação de seqüência do gene UL55 que codifica a glicoproteína gB, o HCMV pode ser classificado em um dos quatro genótipos. Estudos prévios têm investigado a associação destes genótipos a partir de cepas obtidas de pacientes imunossuprimidos. O presente estudo determinou os genótipos gB de cepas de HCMV obtidas de mulheres imunocompetentes em Vitória, Espírito Santo, Sudeste do Brasil. O genoma do HCMV foi extraído de secreção cervical, tecidos placentários fetais e maternos (vilosidade coriônica e decídua) obtidos de casos de aborto e de leucócitos do sangue periférico. A genotipagem foi realizada através da análise de polimorfismo de fragmentos de restrição do produto amplificado da região de alta variabilidade do gene UL55. Todos os quatro genótipos foram detectados na secreção cervical e na placenta, enquanto que somente o genótipo gB1 foi detectado em leucócitos. Genótipos gB1 e

gB2 foram detectados em nove e seis das 23 cepas estudadas, respectivamente, enquanto gB3 e gB4 foram encontrados cada um em quatro casos. A diferença na frequência de genótipos não foi estatisticamente significativa. Infecção mista não foi detectada. Estes resultados indicam que os quatro genótipos de HCMV apresentam tropismo para os tecidos placentários e que todos eles circularam nas mulheres imunocompetentes no período e região geográfica do estudo.

Palavras-chave: citomegalovírus; RFLP; genótipo gB, secreção cervical, placenta

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