Rev. Inst. Med. trop. S. Paulo
46(2):87-92, March-April, 2004

DETERMINATION OF HUMAN CYTOMEGALOVIRUS GENETIC DIVERSITY IN DIFFERENT PATIENT POPULATIONS IN COSTA RICA

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SUMMARY

Seroprevalence of HCMV in Costa Rica is greater than 95% in adults; primary infections occur early in life and is the most frequent congenital infection in newborns. The objectives of this study were to determine the genetic variability and genotypes of HCMV gB gene in Costa Rica. Samples were collected from alcoholics, pregnant women, blood donors, AIDS patients, hematology-oncology (HO) children and HCMV isolates from neonates with cytomegalic inclusion disease. A semi-nested PCR system was used to obtain a product of 293-296 bp of the gB gene to be analyzed by Single Stranded Conformational Polymorphism (SSCP) and sequencing to determine the genetic polymorphic pattern and genotypes, respectively. AIDS patients showed the highest polymorphic diversity with 14 different patterns while fifty-six percent of HO children samples showed the same polymorphic pattern, suggesting in this group a possible nosocomial infection. In neonates three genotypes (gB1, gB2 and gB3), were determined while AIDS patients and blood donors only showed one (gB2). Of all samples analyzed only genotypes gB1, 2 and 3 were determined, genotype gB2 was the most frequent (73%) and mixed infections were not detected. The results of the study indicate that SSCP could be an important tool to detect HCMV intra-hospital infections and suggests a need to include additional study populations to better determine the genotype diversity and prevalence.

KEYWORDS: Human cytomegalovirus; Glycoprotein B; Polymerase chain reaction; Single stranded conformation polymorphism; Sequencing.

INTRODUCTION

Human Cytomegalovirus (HCMV) infection is generally asymptomatic in the immunocompetent individual although the virus persists in the host for life. However, severe infections with different clinical manifestations are common in immunocompromised patients with AIDS or chronic diseases and transplant recipients[19]. In these cases both primary and recurrent infections can induce severe illness, representing a major cause of morbidity and mortality with prolonged hospitalization and high costs[6,26].

HCMV glycoprotein B (gB) gene is considered to be a multifunctional envelope component responsible for virion entry, cell to cell spread, syncytium formation[18] and is the major target for neutralizing antibodies[4,5]. Based on sequence variation of the gB gene, HCMV strains can be classified into four gB genotypes[5] and recently, two new genotypes (gB6 and gB7) have been reported by TRINCADO *et al.*[27]. Genotype variation has been associated with cell tropism, viral pathogenesis and severity of disease. Several reports have suggested an association of different gB genotypes with pathogenicity[6,13,20,24,26,28,29].

A follow-up study carried out in Costa Rica of 131 mothers and their newborn babies from birth to six years of age determined that 46% of the children seroconverted (IgM) during the first three months of life[30]. Another study[12] demonstrated that HCMV was the most common cause of congenital disease, and since seroprevalence in Costa Rica is higher than 95% in adults, it is most likely that this infection is caused by reactivation and/or reinfection of the virus in the mother during pregnancy. Although cytomegalic inclusion disease is more likely in a seronegative pregnant woman who has a primary HCMV infection, HCMV congenital disease can also be found in infants born to women with recurrent disease as described by several authors[3,17,23,25].

To further understand the diversity of HCMV and possible infectious routes, the SSCP has shown to be a valuable tool, since only one base pair (bp) change can affect the DNA Single stranded conformation and therefore its mobility in aclylamide electrophoresis; which reveals the different patterns[8,9,10].

Since infection and reactivation of HCMV is very common in Costa Rica, the objectives of this study were to determine the genetic variability and genotypes of the virus circulating in the country using different study populations.

Grant support: Network for Research and Training in Tropical Diseases in Central America (NeTropica), Vice-Rectory of Research, University of Costa Rica, UCR, Project # 803-A1-531. Louisiana State University-International Center for Medical Research and Training (LSU-ICMRT).

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MATERIALS AND METHODS

Study population: Samples available from different populations for HCMV prevalence studies were used, as follows: 41 Hematology/ oncology (HO) children, 30 AIDS patients, 30 alcoholics, 30 blood donors and 30 pregnant women were collected from 1998 to 2000 by the International Center for Medical Research and Training (ICMRT) and kept at -70 °C. Sixteen HCMV isolates from neonates with congenital infection recovered in 1984 and control strains of genotype gB1 (Towne strain), gB2 (AD-169 strain) and gB3 (isolate from a clinical sample) were also tested. The neonate samples were kept frozen at the Virology Laboratory at the Faculty of Microbiology, University of Costa Rica (UCR) and the control samples were donated by Dr. Mia Britting from the Swedish Institute for Infection and Disease Control, Solna, Sweden. All study samples were assayed by nested PCR and this product was further analyzed by SSCP to determine the genetic diversity. Forty-five samples were selected for the genotype determination representing 23 different polymorphic SSCP patterns (Table 3).

HCMV-DNA extraction: HCMV-DNA was extracted from plasma by the Qiamp Blood kit (Qiagen, Chatsworth, CA) according to manufacture recommendations. DNA samples were re-suspended in 200 µl of water.

Amplification of HCMV-DNA: Polymerase Chain Reaction (PCR) was used to amplify a region of high sequence variability in the gB gene of HCMV as described by AQUINO & FIGUEIREDO[1] with minor modifications. The reaction mixture had a total volume of 50 µl, containing 10 µl of DNA sample, 5 µl of PCR 10x buffer, 2.5 mM of MgCl2, 200 uM each of the dNTPs and 1.6 pM/µl of primers gB1319 (5'TGGAACTGGAACGTTTGGC3') and gB1676 (5'TGACGCTGGTTTGGTTGATG3')[26] and 2.5 U of Taq DNA polymerase (Promega). Amplification of DNA was performed in a thermocycler (Perkin Elmer 9600) with a hot start of 94 °C for 3 minutes, then 10 cycles of 94 °C for 30 sec, 60 °C for 59 sec, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 45 sec, 72 °C for 59 sec and a final extension of 72 °C for 3 min. The amplified products of 357 bp were visualized by electrophoresis in a 2% agarose gel stained with ethidium bromide.

A semi-nested PCR was performed in all samples to obtain an amplified product of 293-296 bp. The reaction mixture contained 1 µl of the first round amplification product using the same concentrations of reagents as described above; but in a final volume of 40 µl. Primers used were 2.1 pM/µl of the gB1319 and 2.25 pM/µl of the gB 1604 primer (5'GAAACGCGCGGCAATCGG3'). Amplification was carried out with 25 cycles at 94 ºC for 30 sec, 55 ºC for 45 sec, 72 ºC for 60 sec, and a final extension of 72 ºC for 3 min.

Single Strain *Conformation* Polymorphism (SSCP): SSCP was performed according to TRINCADO *et al*.[27]. The principle of SSCP is a change in the electrophoretic mobility of the PCR product if there is a bp change that affects its secondary structure. This can be visualized by electrophoresis of a denaturalized PCR product in a polyacrylamide with the appearance of different polymorphic patterns related to the genetic diversity of each sample[8,14]. Five µl of the double-stranded semi-nested PCR product was added to 5 µl formamide buffer (formamide 100%, bromophenol blue 0.02%, xylene cyanol 0.02% and EDTA 10 mM). DNA was denatured at 94 ºC for 10 min, then cooled rapidly on ice; denatured samples were analyzed by electrophoresis on a 7% polyacrylamide gel (BIO-RAD) at 5 watts for 17 hours at room temperature using a vertical water-jacketed electrophoresis system (Life Technologies, Air Cooled System, and S2001). DNA was visualized by silver staining.

HCMV-DNA sequencing: Products of the semi-nested PCR were purified using the Wizard PCR Preps DNA purification Systems kit (Promega) and sequenced by the Big Dye Terminator kit (Applied Biosystems) in a 5% acrylamide gel automatic sequencer (ABI model 373, Applied Biosystems).

The nucleotide sequences were edited by the BIOEDIT programme (www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned using the NCBI (National Center of Biotechnology Information) sequence database.

RESULTS

In PCR 1 a band of 357 bp was visualized and in the semi-nested PCR a band between 293-296 bp was seen (Fig. 1).

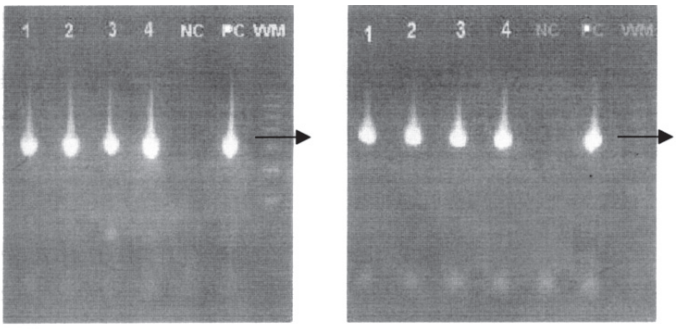

Fig. 1 - Electrophoresis in agarose gel of HCMV gB gene of PCR I products (A) (357 bp) and B PCR II (295-296 bp). Samples 1-4 are HCMV neonate isolations, NC: negative control, PC: positive control (HCMV, AD 169 strain), WM: weight marker.

A total of 53 polymorphic patterns were established in the 180 analyzed samples (Table 1). The group of AIDS patients presented the major genetic diversity with a total of 14 different patterns followed by the group of pregnant women with 10, HO children with 8, alcoholics with 7, blood donors with 6 and HCMV isolates from neonates with 5. Control samples for gB genotypes showed a different polymorphic pattern for each one.

Figure 2 illustrates the results of the HO children, which showed a repetitive polymorphic pattern in 17 of the 30 analyzed samples characterized by the presence of two strong bands at the same positions in the gel. In these samples also weaker bands were observed that varied in intensity with some not being visible. These samples were classified under the same pattern, designed as 1A (Fig. 2). To confirm that this result was not due to contamination during PCR amplification or to the separation of the SSCP in the gel, the samples were re-analyzed, initiating with the extraction of DNA from the original sample. Furthermore, 14 additional samples were included from HO children (Costa Rica) and 5 samples from leukemia children from Nicaragua. The repeated samples all showed the same pattern A1 as well as 5 of the 14 additional samples from the HO children and 2 of the 5 from Nicaragua.

Table 1
Distribution of techniques used to determine HCMV diversity in different study groups

Study group	PCR and SSCP	*Letters	**Pattern No.	Sequenced
HO Children	41	A	1-8	8
AIDS patients	30	B	1-14	7
Alcoholics	30	C	1-7	6
Donors	30	D	1-6	8
Pregnant women	30	E	1-10	6
HCMV isolations	16	F	1-5	7
Reference strains	3	R	1-3	3
Total	180		53	45

*Identification of each study group; **Total polymorphic patterns obtained from each study group

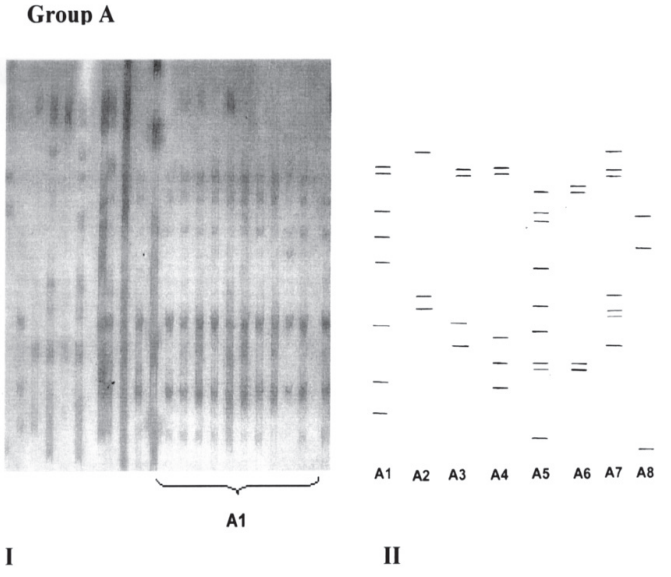

Fig. 2 - SSCP polymorphic patterns of Hematology/Oncology children (Group A), illustrated by the acrylamide electrophoresis bands (I) and the schematic presentation of each pattern (II).

Figure 3 illustrates the diversity of the polymorphic patterns of AIDS patients (bands I and II) and blood donors (III and IV) with 14 and 6 different patterns, respectively.

Of the 45 sequenced samples 37 were successfully genotyped and its distribution within the study groups is shown in Table 2. Of the remaining 8 samples, the genotype could not be determined since sequenced products were not suitable for this procedure.

Of the six analyzed study groups, neonates were the only group that showed HCMV genotypes gB1, gB2 and gB3. Pregnant women showed genotypes gB1 and gB2; HO children and alcoholics showed genotypes gB2 and gB3, while AIDS patients and blood donors only showed genotype gB2. However, great difference between these two last groups was detected regarding frequency of polymorphic patterns with the presence of 14 and 6 patterns, respectively (Fig. 3). The three control sample patterns corresponded to gB1 (Towne strain) gB2 (strain AD169) and gB3 (Table 2). In alcoholics and neonate isolates, two samples with identical polymorphic patterns were found to have different genotypes (Table 3).

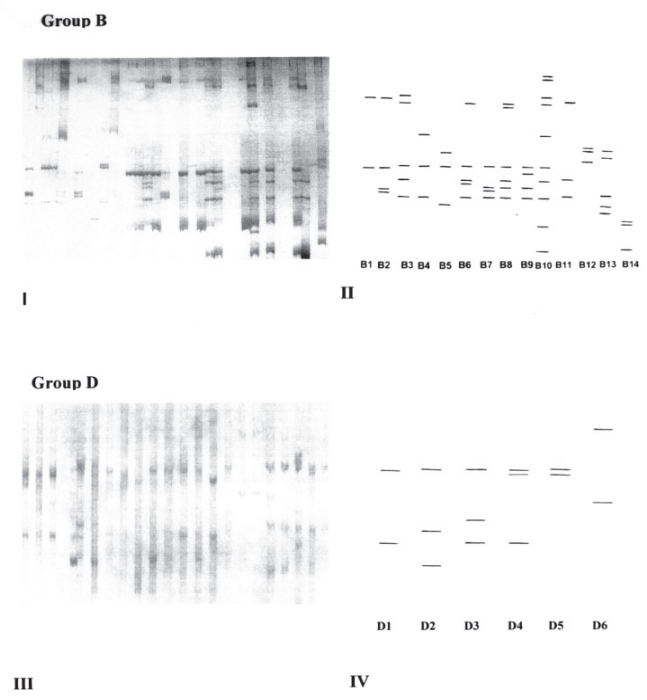

Fig. 3 - SSCP polymorphic patterns of AIDS patients (Group B) and Blood Donors (Group D), illustrated by the acrylamide electrophoresis bands (I and III) and the schematic presentation of each pattern (II and IV), respectively.

The distribution of genotypes in the 37 samples were as follows: 27 samples with gB2 (73%), 6 samples with gB1 (16%), 4 samples with gB3 (11%) and none of these samples had mixed infection. Overall genotype gB2 was the most frequent (73%) and had the greatest genetic variability, followed by genotype gB3 and genotype gB1 (Table 3).

DISCUSSION

A semi-nested PCR method was applied to obtain the largest number of positive samples, since this procedure increase both sensitivity and specificity. SSCP analysis was used to characterize genetic variations among the study populations[9,15] because this method has shown to be both sensitive and simple. A change in nucleotide can easily be detected

Table 2
Distribution of HCMV gB genotypes within the study groups

Study group	*Analyzed samples	Genotypes gB1	gB2	gB3	gB4
HO Children	8		4	1	
AIDS patients	7		4		
Alcoholics	6		4	1	
Donors	8		7		
Pregnant women	6	2	4		
HCMV isolations	7	3	3	1	
Reference strains	3	1	1	1	
Total	*45	6	27	4	

* Of 45 samples sequenced, 37 could be genotyped and 8 were not able to be edited

Table 3
HCMV polymorphic patterns according to genotypes in the different study groups

Study population	Polymorphic patterns	Genotypes
HO children	A1	gB2
	A5	gB3
AIDS patients	B1, B3, B5	gB2
Alcoholics	C1, C2, * C6	gB2
	* C6	gB3
Donors	D1, D2, D3	
	D4, D5, D6	gB2
Pregnant women	E3	gB1
	E1, E2	gB2
HCMV isolations	* F2, F4	gB1
	F1, * F2,	gB2
	F3	gB3
Reference strains	R4	gB1
	R5	gB2
	R6	gB3

* The same letters and numbers identify equal polymorphic patterns

by SSCP, if this alters the secondary structure of the molecule. Therefore, SSCP has the advantage compared to other techniques such as restriction fragment polymorphism (RFP) that it is able to detect variations throughout the amplified product[16]. Its sensitivity depends mainly on the size of the DNA fragment[11] and is decreased when the size is larger than 530 pb[7]. The SSCP fragments in this study were 293-296 bp and 53 different polymorphic patterns were detected in 180 samples.

SSCP has been used for other pathogens (HBV and HCV), showing that from donor to recipient and mother to neonate the SSCP pattern was conserved as well as in patients affected by nosocomial outbreaks in oncology and hemodyalisis units[9,10,11].

In 41 samples analyzed from the HO children using SSCP 24 showed the same polymorphic pattern A1. The hospitalization period among children with patterns different from A1 were relatively short (< 40 days). However, in children with pattern A1, although information regarding hospitalization was incomplete, there was a tendency for it to be over longer periods. Therefore, we speculate that the high frequency of one pattern could be related to nosocomial infections, which is further supported by the fact that the same genotype was established in all samples with pattern A1 (gB2).

The group of AIDS patients had the major amount of polymorphic patterns with one genotype (gB2). This could be related to differences in active replication of HCMV in each of these groups, considering that AIDS patients frequently show pathologies related to HCMV active infection and reactivation/reinfection of immunocompromised patients has been well documented[19]. The AIDS patients results were similar to those reported by BALE *et al*.[2], RASMUSSEN *et al*.[21], ROSEN *et al*.[22], WOO *et al*.[29] in showing the presence of genotype gB2. On the contrary, blood donors showed few polymorphic patterns probably related to a latent disease state, also with only the presence of genotype gB2.

Two situations were observed where different genotypes had the same polymorphic patterns, showing that the difference of bp's related to genotypes did not cause structural changes.

None of the samples had mixed HCMV infections, different from the results reported by AQUINO & FIGUEIREDO[1]. They found different genotypes according to the site of infection and specimen, which suggests a cellular tropism among different genotypes. Therefore, the use of only plasma samples as in this study do not allow us to make final conclusions about the mixed infections in these populations.

In conclusion SSCP method showed a high sensitivity for detection of genetic variations in the samples analyzed (53/180), but maybe even more important was the capacity to identify equal polymorphic patterns that relates to the same origin and therefore could be an indication of nosocomial infection. The results obtained show the need to carry out further studies focusing on a possible nosocomial infection at the National Children's Hospital, to determine if strain A1 is of dominant circulation within the hospital and also to evaluate its possible resistance to the antiviral drugs currently in use.

The data show the predominance of HCMV genotype gB2 in Costa Rica among the study groups, with its limitations regarding the number of total individuals analyzed and the type of samples used (only plasma). In the future this study should be amplified including a more diverse study population and collection of different specimens from each participant with an approach of longitudinal studies.

RESUMEN

Determinación de la diversidad genética del citomegalovirus humano en diferentes poblaciones de pacientes en Costa Rica

La seroprevalencia de citomegalovirus es mayor del 95% en la población adulta de Costa Rica; la infección primaria ocurre muy temprano en la vida y es la infección congénita más frecuente en recién nacidos. El objetivo de este trabajo fue determinar la variabilidad genética y los genotipos del gene gB del citomegalovirus humano. Se recolectaron muestras de sangre de mujeres embarazadas, alcohólicos, pacientes con

SIDA, niños con trastornos hemato-oncológicos, donadores de sangre y se incluyeron aislamientos de citomegalovirus de neonatos con enfermedad congénita. Se utilizó un sistema de PCR semi-anidado para obtener una banda de 293-296 pares de bases, la cual fue analizada por la técnica de Polimorfismo conformacional de banda simple (PCBS) y secuenciada para determinar los patrones genéticos polimórficos y los genotipos, respectivamente. La mayor diversidad polimórfica se encontró en los pacientes con SIDA con 14 patrones diferentes mientras que en los niños con trastornos hemato-oncológicos se demostró el mismo patrón en el 56% de los casos, sugiriendo una posible infección nosocomial en este grupo. En los neonatos se encontraron tres genotipos (gB1, gB2, gB3) mientras que en los pacientes con SIDA y en los donadores de sangre solo se demostró el gB2. En las muestras analizadas se determinaron los genotipos gB1, gB2 y gB3 y el gB2 se determinó en el 73% de los casos, no se detectaron infecciones mixtas. Los resultados de este trabajo indican que la técnica del PCBS puede ser una herramienta importante para detectar el citomegalovirus humano en infecciones intrahospitalarias y se sugiere la importancia de incluir poblaciones de estudio adicionales para determinar mejor la diversidad genética y su prevalencia.

ACKNOWLEDGEMENTS

The authors thank The Costa Rican Social Security System, the Institute of Alcoholics and Drug Abuse, Costa Rica and Dr. Mia Britting from the Swedish Institute for Infection and Disease Control, Sweden, who kindly facilitated the study and reference samples, respectively. Andreas Busse, MS from the Center of Investigation in Molecular and Cell Biology, UCR and Carlos Vargas from the LSU-ICMRT for technical assistance. Ms. Virginia Larrad from the LSU-ICMRT for supporting the preparation of the manuscript and Mrs. Xinia Arias from the Faculty of Microbiology, UCR for secretarial support.

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Received: 4 September 2003
Accepted: 8 March 2004