RESEARCH PAPER

Glycoprotein B Genotyping in Congenital/perinatal Cytomegalovirus Infection in Symptomatic Infants

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Background: Molecular epidemiological studies on circulating strains of CMV in cogenital/perinatal infections have not been done earlier in this region.

Objective: To study the glycoprotein B genotypes in babies with symptomatic congenital/perinatal CMV infection and to assess the possible influence of genotype on the outcome of the infection.

Methods: Clinical samples (blood and urine) of symptomatic babies are sent to the Virology Department of NCDC, Delhi for the diagnosis of congenital infections. 375 clinical samples of infants (newborn - 6 months old) were included for the study. Serum samples were subjected to ELISA for detection of IgM antibodies against CMV. DNA isolation and amplification of CMV genomic DNA targeting gB gene fragment by nested PCR, was carried out in the samples. The amplified fragment including the cleavage site was subjected to RFLP using restriction enzymes Rsal and Hinf1. They were also verified by sequencing using Big Dye Terminator chemistry**.**

Results: 75 samples out of 375 tested were confirmed positive for CMV infection by serology and PCR. Both RFLP and sequencing of gB gene fragment showed that gB 1, 2 and 3 genotypes were in circulation. gB 3 was the most prevalent genotype in symptomatic infants. Hepatosplenomegaly was the most common feature in gB-3 genotype of CMV. gB2 congenital CMV infection was more commonly associated with long term sequelae.

Key words: Cytomegalovirus, Epidemiology, Glycoprotein B, gB genotypes, India,.

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Globally; CMV has emerged as the most important cause of congenital infection, in recent years. Congenital CMV infection may lead to hearing, cognitive and motor impairment in babies [1,2].

The large CMV genome encodes several hyper variable loci. Many genetically different strains of CMV circulate in the human population. It has been suggested that difference in virulence, pathogenicity, progression and severity of disease in immunocompromised individuals, including transplant recipient may be attributed to variation between HCMV strains. Considerable attention has recently been focused on the analysis of strain variation among HCMV isolates. Some 20 different strains have been isolated and differentiated by restriction analysis of PCR amplified DNA fragments [3-5]

Glycoprotein B gene of Cytomegalovirus plays an important role in virus infectivity, cell to cell spread and is a major target for antibody mediated immunity [6,7]. There are several variable regions of gB and CMV strains have been classified into four genotypes based on restriction fragment length polymorphism(RFLP) of a fragment corresponding to the cleavage site between residue 460 and 461 [5,8]. These genotypes have been reported to be clinically associated with the outcome of the disease though these reports are contradictory [9-12]. Recently evidence of new strains arising in individuals infected with multiple CMV strains and intragenic variations within gB gene were obtained [9,10].

Few sero-epidemiological studies conducted in Indian population, have shown that there is prevalence of 80-97% seropositivity of CMV antibodies (IgG) in women of child bearing age [13-15]. High incidence of congenital CMV infection in babies due to in-utero CMV infection in mothers by reactivation/reinfection or primary infection of the virus has also been reported besides perinatal infections acquired by newborns [15-18]. The magnitude of the problem, in India has not been systematically investigated. The present study was undertaken to determine the prevalence of congenital CMV infection in symptomatic babies and the gB genotyping of CMV strains circulating in the affected babies. An attempt was

made to associate the possibility of clinical and prognostic significance of the prevailing genotypes.

METHODS

Three hundred seventy five clinical samples (blood and urine) from 375 infants (newborn to 6 months old) babies exhibiting various symptoms of congenital infection, referred to Virology laboratory of the National Centre for Disease Control were selected for the present study. The samples were accompanied by duly-filled proforma with relevant history and clinical details . All the samples were negative for HIV, and without any history of blood/blood product transfusion. Blood and urine samples from 60 asymptomatic babies, referred to NCDC for other testing were included in the study and treated as control group.

Blood samples were clotted and centrifuged for serum separation prior to testing. All the sera were stored at -20ºC pending testing. Serum samples of the babies were tested for CMV-IgM antibodies using μ-capture ELISA (RADIM). DNA was isolated from urine samples and serum samples by QIamp DNA Mini kit (QIAGEN, Germany) as per manufacturer's protocol. Fragment from gB (UL55) gene region was amplified from isolated DNA samples using pre-published DNA oligonucleotide primers [19], by nested PCR. Primers for Outer-PCR: gB-1 CAAGARGTGAACATGTCCGA, gB-2 GTCACGCAGC TGGCCAG. Thermal Cycling Profile: Initial Denaturation 95ºC: 10 min; Amplification:35 Cycles: Denaturation 95ºC : 1 min; Annealing 55°C 1min ; Extension 72ºC:1 min; Final Extension 72ºC:7 min.; Inner-PCR: gB-3 TGGAAC TGGAACGTTTGGC, gB-4 GAAACGCGCGGC AATCGG. Thermal Cycling Profile: Initial Denaturation 95ºC 10 min; Amplification: 35 Cycles: Denaturation 95°C: 30 seconds; Annealing: 54ºC: 45seconds; Extension 72ºC: 30seconds; Final Extension 72°C: 7 min.

The PCR products (520bp, 305bp) were subjected to gel electrophoresis on 1.5% agarose to visualize the product with ethydium bromide stain. The inner amplified products from gB gene region were digested with the Restriction Enzymes, Hinf1 and Rsal (Promega Madison, Wise, USA). Approximately 1 μg of the PCR product was added to 2 μL of PCR buffer and 1unit of enzyme, mixed gently, spinned briefly and incubated at 37°C for 3 hrs. for digestion. The digested fragments were analyzed on 3% agarose gel. The 50 bp DNA ladder was also loaded on gel to compare the fragment length of the digested products. Distinct gB genotypes could be identified by the different lengths of restriction fragments (***Table* I**). Sequencing was also carried out from the inner (305bp) PCR product using Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, USA).

Representative sequences derived from this study were submitted to Genbank at NCBI website and accession numbers acquired. Bioinformatics software viz. BLAST, CLUSTALW, BIOEDIT, MEGA v.4 were used for sequence analysis.

TABLE I FRAGMENT LENGTH (BP) OF FOUR GB GENOTYPES IDENTIFIED BY RFLP ANALYSIS WITH HINF1 OR RSAL DIGESTION

	gB-1	*gB-2*	*gB-3*	*gB-4*
HinfI	202,67,36	202,100	202,97	202,67,36
Rsal	239,66	239,63	195,63,41	195,66,44

RESULTS

Among the clinical manifestations displayed by the babies, Hepatosplenomegaly was the most common feature (55.3%) followed by neonatal jaundice, broncho-pneumonia, developmental delay, neonatal cholestasis, micocephaly and congenital cataract. Hearing impairment, hydrocephaly, and chorioretinitis were also present in some infants (***Fig.* 1**).

The high incidence of congenital CMV infection (19.4%) was detected among babies born with various birth defects. The maximum number of symptomatic babies presented to the hospitals in the age group of (O-1M) and the case reporting decreased in the subsequent months. Positivity rate was highest in the age group (1+ to 2 months) (28%) (***Table* II**). Higher number of male infants (64%) were positive. All the samples of babies from the control group were negative for congenital CMV infection by ELISA and PCR.

RFLP of gB (UL-55) PCR products for genotyping demonstrated prevalence of gB [1-3] genotypes in the babies. gB 4 was not detected (***Fig.* 2**). The frequency of gB3 was the most dominant *(49.25%)* followed by gB1 (24.4%) and gB2 (22.4%).

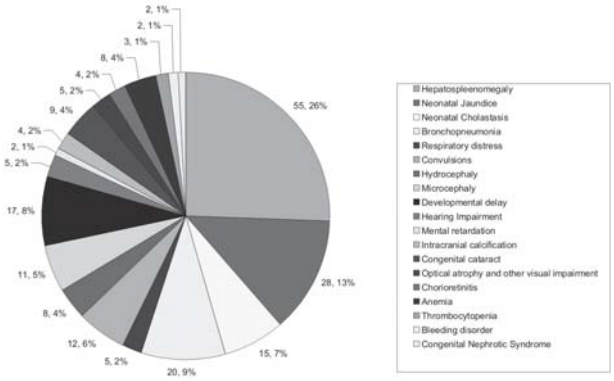

FIG. 1 *Clinical features in babies with congenital/perinatal CMV Infection.*

TABLE II INFANTS POSITIVE FOR ANTI CMV-IGM ANTIBODIES (*N*=75)

Age group(mo)	No. of babies		Total (%)
	Male	Female	
NB-1	6	4	10 (13.3%)
1 + to 2	15	6	21 (28%)
2 + to 3	13	7	20 (26%)
3 + to 4	9	6	15 (20%)
4 + to 5	3	2	5 (6.67%)
5 + to 6	2	2	4 (5.33%)

Male : Female *48 (64%) : 27 (36%)*

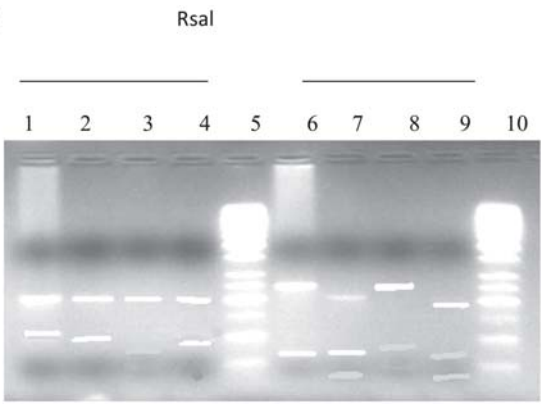

FIG. 2 *Restriction enzyme analysis of gB genotype by Hinf1 & Rsal. Lane-1,6: gB-2; Lane-2,4,7,9 : gB-3; Lane-3,8 : gB-1; Lane-5,10: Molecular markers of 50 bp*

GenBank accession numbers of the representative submitted sequences for gB gene region from this study are: EU938342 to EU938348; HM069142 to HM069157. Sequences from variable region of gB gene (cod 448 to 480) including proteolytic cleavage site were compared to the published sequences of 4 gB genotypes. The result from the sequencing was concordant with RFLP results.

The prevailing gB genotypes and specific CMV disease manifestations in infants were compiled to analyse the existence of correlation with pathogenesis of the disease. It was found out that liver disorders were mainly associated with gB3 genotype, and hearing impairment and central nervous system symptoms with genotype gB2 (***Table* III**).

DISCUSSION

A high incidence of congenital CMV infection (19.4%) was detected among babies born with various birth defects, similar to our previous study and other studies conducted in India and globally [15-17,20,21]. The higher number of positive cases in the age group (1+ to 2 months) (28%), could be due to the fact that excretion of CMV virus and antibody development is mostly detected after 2-3 weeks of birth in congenital CMV infection besides cases of perinatal infection.

In the present study clinical samples from the patients were processed for diagnosis and molecular studies to avoid any variations which might occur during cell culture and subcultures. Clinical samples were sufficient to carry out testing and retesting of the samples.

RFLP of gB (UL-55) PCR products for genotyping and sequencing showed that only 3 genotypes (*gB1, gB2 and gB3*) were prevalent in the babies. Some studies from other countries have also reported prevalence of these 3 genotypes in infants with congenital CMV infections [22, 23]. No data on gB genotyping in babies with congenital CMV infection is available from India though this kind of study in immunocompromised patients have been conducted earlier [24, 25], in which the prevalence of all 4

TABLE III GB GENOTYPES DISTRIBUTION IN 67 BABIES WHO WERE POSITIVE FOR CONGENITAL/PERINATAL CMV INFECTION

gB genotypes/ Clinical manifestations	gB-1	gB-2	gB-3	Failure
Hepatosplenomegaly, NNcholestasis, jaundice, pneumonia and respiratory distress, developmental delay with other clinical features like anemia, thrombocytopenia	9	1	26	-
Clinical features involving CNS and long term seuelae (Microcephaly, hydrocephaly, hearing impairment, intracranial calcification, mental retardation etc.) with other secondary clinical features of respiratory disorders.	1	10	2	-
Congenital cataract, chorioretinitis, optical atrophy and other visual impairments with many other anomalies like develop-mental delay, hepatospleenomegaly,anemia,pneumonia etc.	7	4	5	2
Total	17	15	33	2

genotypes and mixed infection of gB genotypes has been documented. No mixed infection was seen in this study, but in two digested PCR products, the fragment pattern could not be identified. Among the prevailing genotypes the frequency of gB3 was the most dominant (49.25%). The findings from the present study are different from some other studies from China and South Hungary, where they have found gB1 as the most dominant genotype circulating in babies with congenital CMV infection [23, 26, 27]. Various clinical studies have suggested that gB genotype of HCMV strains may influence the clinical outcome of acquired CMV infection [9-11]. Earlier global studies have shown that gB1 genotype is associated with hepatospleenomegaly [23,26]. The new feature demonstrated by the present study was that babies who had manifestation of hearing impairment and symptoms with CNS association had mainly genotype gB2 infection. It has not been reported in any other study done on congenital CMV infection. However, this finding needs to be explored further as the sample size was small in this study.

To date, most knowledge regarding the medical implications of viral disease stems from studies of acute viral infections. Symptomatic congenital CMV infection is a significant cause of morbidity in developing as well developed countries like United States. It has been estimated that direct and indirect costs for treating babies with congenital CMV infection approaches $1 billion dollars per year[28]. As a consequence, prevention of this disease has become a global issue for vaccine development, particularly for administration to seronegative susceptible women of child-bearing age [29, 30]. A virus such as CMV, which is able to establish latency and evade immune surveillance, presents particular challenges in the development of effective vaccination as HCMV genome displays great genetic heterogeneity. Thus, there is a constant need for upgrading the information on molecular epidemiology of CMV in different population, which would help in forming efficacious vaccines for the prophylactic treatment of CMV in humans.

Glycoprotein B (gB) is a major target for neutralizing antibodies and an important component of recombinant vaccines, under trial. Therefore, more studies on genetic variability data in gB gene, from India may help to determine the optimal strains for vaccine development in Indian population

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REFERENCES

1. Friedman S, Ford-Jones EL. Congenital cytomegalovirus infection: an update. Pediatr Child Health.1999;4:35-8.
2. Benjamin Bar-Oz, Berkovitch M, Lee Ford-Jones, Koren G. Congenital cytomegalovirus infection: Is there a breakthrough? Canadian Family Physician. 2001;47: 1179-81.
3. Grillner L, Ahlfors K, Ivarsson SA, Harris S, Svanberg L. Endonuclease cleavage pattern of cytomegalovirus DNA of strains isolated from congenitally infeced infants with neurologic sequelae. Pediatrics. 1988;81:27-30.
4. Peckham CS, Garrett AJ, Chin KS, Preece PM, Nelson DB, Warren DE. Restriction enzyme analysis of cytomegalovirus DNA to study transmission of infection. J Clin Pathol. 1986;39:318-24.
5. Chou S. Differentiation of cytomegalovirus strains by restriction analysis of DNA sequences amplified from clinical specimens. J Infect Dis. 1990;162:738-42.
6. Britt WJ, March M. Human cytomegalovirus proteins. Intervirology. 1996:39:401-12
7. Navarro D, Lannette C, Tugizov S, Pereira L. Humoral immune response to Functional Regions of human cytomegalovirus Glycoprotein BJ Med Virol. 1997;52:451-9.
8. Chou SW, Denninson KM. Analysis of interstrain variation in cytomegalovirus glycoprotein B sequences encoding neutralization-related epitopes. J Infect Dis.1991; 163:1229-34.
9. Shepp DH, Match ME, Ashraf AB, Lipson SM, Millan C, Pergolizzi R. Cytomegalovirus glycoprotein B groups associated with retinitis in AIDS. J Inf Dis. 1996;174: 184-7.
10. Trincado DE, Scott GM, White PA, Hunt C, Rasmussen l, Rawlinson WD. Human cytomegalovirus strains associated with congenital and perinatal infections. J Med Virol 2000;61:481-7.
11. Fries BC, Chou S, Boeckh M, Torok-Storb B. Frequency distribution of cytomegalovirus envelope glycoprotein genotypes in bone marrow transplant recipients. J Infect Dis. 1994;169:769-74.
12. Vogelberg C, Meyer-Koni U, Hufert FT, Kirste G, Von Laer D. Human Cytomegalovirus glycoprotein B genotype in renal transplant recipients. J Med Viro. 1996;50:31-34.
13. Rai A, Kumari S, Khare S, Gandhoke I., Bhatia R, Datta KK. Maternal viral infections and their implications in congenital defects of new borns. J Basic and Applied Biomedicine. 1995;3:1-9.
14. Jindal S, Aggarwal N. A pilot seroepidemiological study of cytomegalovirus infection in women of child bearing age. Indian J Med Microbiol. 2005;23:34-6.
15. Chakravarty A, Kashyap B, Rathi K. The seroepidemiological study on cytomegalovirus in women of child-bearing age with special reference to pregnancy and maternal-fetal transmission. Indian J Pathol Microbiol. 2005;48:518-21.
16. Abraham M, Abraham P, Jana AK, Kuruvilla KA, Cherian T, Moses PD, Mathai E, .John TJ, Sridharan G.

Serology in congenital infections: experience in selected symptomatic infants. Indian J Pediatr. 1999;36:697-700.
17. Gandhoke I, Aggarwal R, Lal S, Khare S. Congenital CMV infection in symptomatic infants in Delhi and surrounding areas. Indian Journal of Pediatrics. 2006;73:1095-7.
18. Rekha S, Chandrasekhra MK, Yeshwanth M. Cytomegalovirus infection acquired through blood transfusions. Indian Pediatr. 1995;32:575-7.
19. Aldo De Albuquerque Cunha, Lauro Juliano Marin, Victor Hugo Aquino, Luiz Tadeu Moraes Figueiredo. Diagnosis of cytomegalovirus infections by qualitative and quantitative pcr in hiv infected patients. Rev Inst Med Trop S Paulo. 2002;44:127-32.
20. Ho M. Cytomagalovirus. *In*: Mandell GL, Douglas RG, Bennett JE. (*Eds*.) Principles and Practice of Infectious Diseases. Third Edition.Chuchill Livingstone, New York, 1990, p. 1159-72.
21. Ahlfors K, Ivarsson SA, Harris S. Svanberg L, Holmqvist R, Lenmark B, *et al*. Congenital cytomegalovirus infection and disease in Sweden and the relative importance of primary and secondary maternal infections. Scand J Infect Dis. 1984;16:129-38.
22. Ahumada-Ruiz S, Taylor-Castillo S, Visona K, Luftig RB, Herrero-Uribe L. Determination of human cytomegalovirus genetic diversity in different Patient Populations in Costa Rica. Rev Inst Med Trop S Palo. 2004;46:87-92.
23. Zhong Sheng, Chao Chun Zou, Ji Yan Zheng, Yan Zhao. Cytomegalovirus gB genotypes and clinical features in Chinese Infants with Congenital Infections. Intervirology. 2006; 49:281-5.
24. Sowmya P, Dhanya V, Madhavan HN, Therese KL. Comparative efficacy of PCR-based restriction fragment length polymorphism (RFLP) and multiplex PCR for glycoprotein B (gB) genotyping of human cytomegalovirus. Indian J Med Res. 2007;126:122-7.
25. Novak Z, Ross SA, Patro RK, Pati SK, Kumbla RA, Brice S, *et al*. Cytomegalovirus strain diversity in seropositive women. J Clinical Microb 2008; 882-6
26. Lukácsi A, Tarodi B, Endreffy E, Bábinszki A, Pál A, Pusztai R, *et al*. Human cytomegalovirus gB genotype 1 is dominant in congenital infections in South Hungary. J Med Virol. 2001;65:537-42.
27. Barbi M, Binda S, Carropo S, Primache V, Didò P, Guidotti P, *et al*. CMV gB genotypes and outcome of vertical transmission: Studies on dried blood spots of congenitally infected babies. J Clin Virol. 2001;21:75-9.
28. Manning FJ, Swaetz M (*Eds*.) Clinical Trials. Review of the fialuridine (FIAU) clinical trials: National Academy Press. 1995.
29. Gonczol E and Plotkin S. Development of a cytomegalovirus vaccine: lessons from recent clinical trials. Exp Opin Biol Therap. 2001;1:401-12.
30. Pas RE, Burke RL Development of Cytomegalovirus Vaccines: prospects for prevention of congenital CMV infection. Semn Pediatr Infect Dis. 2002;13:196-204.