Cytomegalovirus Glycoprotein B Genotypes Distribution among HIV Positive Patients on Highly Active Antiretroviral Therapy in Bida, North Central Nigeria

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ABSTRACT

OBJECTIVE: Cytomegalovirus is considered to be one of the most serious pathogens affecting immunosuppressed individuals particularly HIV/AIDS and glycoprotein B (gB) is essential for viral infectivity as they play a role in attachment and penetration of the host cells during viral transmission and fusion into infected cells. This study was conducted to determine the molecular diversity of gB glycoproteins of CMV strains distribution among HIV positive patients in Federal Medical Centre Bida. **METHODS**: Blood samples were collected for IgM ELISA and $CD4^+$ cell counts. Viral DNA extraction was carried out on all IgM positive samples and genotyping of glycoprotein B gene (UL 55 region) was detected by multiplex nested PCR. **RESULTS**: Out of the three hundred and eighty five (385) HIV positive subjects, a prevalence of 19.8% CMV IgM was found among patients with HIV in Bida. PCR revealed that gB1 was more prevalent (83.7%) followed by gB2 (16.3%) among subjects. The absence of mixed infection confirmed that there was no case of reinfection as all the primary infections were cases of reactivation of CMV infection among HIV patients.

CONCLUSION: This study found significant relationship in marital status, location and educational status with gB genotype distribution in Bida. Although, there was paucity of reports on CMV gB genotype distribution with socio demographic factors and behavioral data, this study provided baseline information on CMV molecular diversity of CMV genotypes gB1 and gB2 among HIV positive patients in Nigeria, more studies on CMV glycoprotein B genotype from Nigeria may help to determine the optimal strains for CMV vaccine development in Nigerian population.

Keywords: **Cytomegalovirus, Glycoprotein B, HIV/AIDS, HAART, Bida.**

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INTRODUCTION

Human Cytomegalovirus infection is endemic and occurs throughout the year, it has no seasonal pattern. About 40-80% of individuals are seropositive at puberty though an early childhood infection (1). CMV a member of the human Herpes family of viruses, transmissible through blood component, transfusion is an important cause for concern worldwide this is because the majority of adults have serological evidence of previous infection. (2).Clinical manifestation occurred in 25-40% of AIDS patients before the starts of Highly Active Antiretroviral Therapy (HAART), (3, 4, 5) all reported that the incidence of CMV disease declined rapidly and significantly by use of HAART. In these patients, end organ disease frequently occurred as a reactivation of latent virus in CMV positive patients .However, CD_4^+ levels less than 50cells/mm^3 are good indicators in the prognosis of clinical manifestation of CMV (6).

CMV infections and reinfection are detectable from clinical samples through isolation of the virus in tissue culture, by genotyping based on variations in different genes, such as the *UL55* gene that encodes CMV glycoprotein B, or by analyzing strain-specific antibody responses to epitopes of different proteins, such as envelope proteins gH (AP86, TO86) and gB (AD55, TO55) of different strains such as the AD 169 and Towne (7) For epidemiological studies, the analysis of antibody responses to CMV strains are preferred when investigating reinfection in cohorts, since viral shedding may be limited and intermittent, detection depends on the biologic fluid examined (8)

The aim of this work is to study the molecular diversity of gB glycoprotein of CMV strains and socio-demographic distribution of gB strain circulating among primary CMV infection in HIV/AIDS patients attending Anti-Retroviral Therapy Clinic in Federal Medical Centre, Bida Niger State Nigeria.

MATERIALS AND METHODS

This study was a cross sectional and epidemiological study of patients with HIV infection attending the Anti-Retroviral Therapy (ART) Clinic of Federal Medical Centre (FMC), Bida, Niger State. The study was carried out in the Anti-Retroviral Therapy (ART) Clinic of Federal Medical Centre Bida, located in Bida Local Government Area of Niger State, North Central Nigeria.

Approval for this study was obtained from the Ethical Review Committee of FMC Bida (FMCB/HCS/HREC/APPR/VOL1/5/15).The sample collection was explained to the subjects using the information sheet prepared by the researcher. Each subject was required to give a written informed consent before being eligible to participate in the study. The minimum sample size for this study was determined using the Fischer formula. (9)

SAMPLE SIZE DETERMINATION

The minimum sample size for this study was determined using the Fischer formula. (Fischer *et al*., 1998)

Using this formula:

$$\frac{Z^2pq}{d^2}$$

n = the desired sample size when population is greater than 10,000

z = the standard normal deviated, usually set at 1.96 (or 2.0) which corresponds to the 95% confidence limit.

P = the proportion in the target population estimated to have a particular characteristic which is 50%

P = 0.5.

q = 1.0 – p

q = 1- 0.5 = 0.5

d = degree of accuracy desired usually set at 0.05

Thus if the proportion of target population which contains the characteristic is 0.05, the z statistic is 1.96 and the desired sample size will be calculated thus:

$$n = \frac{Z^2 pq}{d^2}$$

$$n = \frac{1.96 \times 1.96 \times 0.5 \times 0.5}{0.0025}$$

n = 384.16

Since the population is less than 10,000

nf = n/ [1+ (n-1/N)]

Where nf = the desired sample size when population is less than 10,000

n = the desired sample where population is greater than 10,000

N = the of the population size. Estimated number of subjects at the ART clinic for the year 2014 is 4000. (FMC Bida ART Patients record)

Therefore nf = 384.16/ [1+ (383.16/4000)]

Minimum sample size for this study is 351.

Five milliliters of whole blood was drawn from the median cubital vein of every volunteer into a plain tube, allowed to clot; specimen shall be processed into serum by room temperature centrifugation at 3000 revolution per minutes for 10 minutes and stored at -20^{0}c for ELISA. Two milliliters of blood was collected in EDTA for CD_4^+ counts estimation.

The frozen serum was thawed at room temperatures for 45 mins. Enzyme Linked Immonosorbent Assay (ELISA) IgM DIA Source (KAPRCVG01) CMV IgM kit was used following manufacturer instruction (RD-Ratio Diagnostics , Germany)

Glycoprotein B Genotypes by Multiplex Nested PCR Assay

Gene extraction

Viral DNA were extracted from plasma samples by Quick-g DNA™ Mini Prep Kit (ZYMO RESEARCH CORP. USA) and the genomic DNA concentration and purity were determined by using the Nanodrop 1000 (ThermoScientific UK) The oligonucleotides primers were supplied by Inqaba Biotechnical Industries Ltd, South Africa and which were used for multiplex PCR and included cytomegalovirus glycoprotein B gene (UL55) are described by (10).

HCMV gB genotype was performed by multiplex nested PCR using a mixture of specific primers to each of gB types., For detection of different gB genotypes, nested multiplex PCR was performed with two external primers and five upstream inner primers specific for each gB genotype (gB-1, gB-2, gB-3, gB-4, and gB-5) and a one downstream primer as described by (10).

Table 1: Primers used for this study

Primers	Primer sequences	Amplicon length
First round:		751 bp
Forward CMV Q1+	5’ TTT GGA GAA AAC GCC GAC3’	
Reverse CMV Q1-	5’CGC GCG GCA ATC GGT TTG TTG TA3’	
Second round:		
Forward primers		
CMV GT1+(gB1)	5’ATG ACC GCC ACT TTC TTA TC3’	420 bp
CMV GT2+(gB2)	5’ TTC CGA CTT TGGA AGA CCC AAC3’	613bp
CMV GT3+ (gB3)	5’TAG CTC CGG TGT GAA CTC C3’	190bp
CMV GT4+(gB4)	5’ ACC ATT CGT TCC GAA GCC GAG GAG TCA 3’	465bp
CMV GT5+ (gB5)	5’ TAC CCT ATC GCT GGA GAA C3’	139bp
Common reverse Primer CMV Q2-	**5’ GTT GAT CCA CAC ACC AGG C 3’**	

Primer sequences of multiplex nested PCR for glycoprotein B (UL55) gene of HCMV

The first round of the nested multiplex PCR was carried out in a 50 µl reaction volume using 1 µl of each external upstream and downstream primers (10 pmol/ µl), 5 µl of purified DNA, 25 µl GoTaq green Master Mix 2X (Inqaba Biotechnical Industries Ltd, South Africa.) and 18 µl of nuclease free water (Biolabs, England). The PCR (ABI 9700 Applied Biosystems) thermal profile started with an initial denaturation 94°C for 5min, followed by 35 cycles at 94°C for 45s ,60°C for 1 min and 72°C for 45 S followed by terminal extension at 72°C for 10 min. The second round of PCR was performed using 5 µl of the first amplified products as DNA template and an equimolar mixture of (10 pmol/ µl) of each inner primer in a 50 µl total volume. Reaction was carried out under conditions identical to those used in the first reaction, but the annealing temperature was 58 °C instead of 60 °C.

Agarose gel electrophoresis of PCR products

Ten µl of amplified PCR products were analyzed on 2 % Agarose gels (Bio-Rad/ USA) stained with ethidium bromide and viewed under UV trans illuminator. The amplified products size was determined by comparing with the reference DNA molecular weight marker (Ladder), in this study 200-1000 bp Ladder was used (Biolabs ,England) .The results generated from this study were analyzed by the statistical software SPSS version 20 (SPSS Inc. Chicago. Illinois).

RESULTS

Three hundred and eighty five (385) blood samples were collected among patients with HIV/AIDS attending ART clinic in Federal Medical Centre Bida. Subjects in this study were within the ages of less than 1year to70 years with a mean age of 37.04 $\pm$ 1.904 years. The prevalence of CMV IgM study report among HIV subjects in Bida was presented in Table 2. Of the three hundred and eighty five (385) HIV seropositive subjects, 77(19.8%) were positive for anti-CMV IgM antibodies.

The distribution of CMV genotypes in HIV subjects revealed that of the 43 positive cases, 36 (83.7%) were of gB1 genotype while 7(16.3%) were gB2 . The prevalence of gB1 genotype was higher among HIV subjects (Table 3)

Gender distribution of CMV genotypes among HIV/AIDS is presented in Table 4 in Bida. Out of the overall gB1 and gB2 genotypes from this study, gB1 genotype had a higher distribution in females (55.1%) while (47.4%) was found in males. gB2 genotype had a higher distribution of (15.4%) in males and (8.2%) in females. There was not statistical significant difference between gender and gB genotypes of CMV (p = 0.767)

Age distribution of CMV genotypes among HIV/AIDS subjects is presented in Table 5. The highest distribution of (100%) was found among age group 10-19 years followed by age group 50-59 years (77.8%) and 40-49 years (53.3%) in gB1genotype, while gB2 genotypes had the highest distribution of (33.3%) in 0-9 years followed by (23.1%) in 20-29 years .There was no statistical significant difference (p = 0.244) between age and the CMV genotype distribution.

Distribution of CMV genotypes among HIV subjects based on location of residence is presented in Table 6. The distribution of gB1 genotype was higher among participants from the rural settings (72.2%) than the urban (46.0%), while the distribution in gB2 was higher in the participants from the urban setting (12.0%) than the rural (5.6%).There was a significant difference (p = 0.021) in the outcome.

The distribution of CMV genotypes among HIV subjects based on marital status is presented in Table 7. The distribution

among gB1 was higher in married (53.2%) than their singles (52.9%) counterparts. While the distribution in gB2 was higher in singles (23.5%) than the married (6.4%).The study found statistical significant difference (p = 0.040) among various marital groups.

The distribution of CMV genotypes outcome among the subjects based on educational status is presented in Table 8. The distribution among gB1 was highest in secondary school education (57.1%) followed by primary education (55.6%) and tertiary education (45.5%). While the distribution in gB2 was highest in primary education (27.8%) followed by tertiary education (4.5%) and secondary education (3.6%). There was significant difference (p= 0.003) in this study.

$CD4^+$counts distribution of Cytomegalovirus gB genotypes among HIV subjects is presented in Table 9. The distribution of gB1 genotypes was the same among subjects with $CD4^+$ count <200 cells/µl and $CD4^+$ count >200 cells/µl (52.9%). While the distribution of gB2 genotypes was higher among subjects with $CD4^+$ count <200 cells/µl (11.8%) than subjects with $CD4^+$ count >200 cells/µl (9.8%). There was no significant difference (p = 0.426).

Table 2: Prevalence of Cytomegalovirus infection among HIV patients in Bida.

Methods	No tested	No positive (%)
Serology (IgM)	**385**	**77(19.8%)**
Molecular (PCR)	**68**	**43(63.2%)**

Table 3: Molecular Diversity of the gB Glycoprotein of CMV Strains Distribution among HIV Positive Patients on HAART in Bida

CMV gB genotype	Frequency	Percentage
gB1	36	**83.7%**
gB2	7	**16.3%**
Total	**43**	**100%**

Table 4: Gender Distribution of CMV gB Glycoprotein Genotypes among HIV Positive Patients on HAART in Bida

Gender	No. Tested	gB1 no (%)	gB2 no (%)	95% CI
Male	19	9(47.4%)	3(15.4%)	**0.475, 1.031**
Female	49	27 (55.1%)	4 (8.2%)	
n= 43	**68**	**36 (83.7%)**	**7(16.3%)**	

***p* - value 0.767**

Table5: Age distribution of CMV gB Glycoprotein Genotypes among HIV Positive Patients on HAART in Bida

Age (Years)	No. Tested	gB1 no. (%)	gB2 no. (%)	95% CI
0-9	3	1 (33.3%)	1 (33.3%)	**1.697, 2.442**
10-19	4	4 (100.0%)	0 (0%)	
20-29	12	4 (33.3%)	3 (25.0%)	
30-39	25	12 (48.0%)	2 (8.0%)	
40-49	14	8 (57.1%)	1 (7.1%)	
50-59	9	7 (77.8%)	0 (0%)	
60-69	1	0 (0%)	0 (0%)	
70	0	0 (0%)	0 (0%)	
n= 43	**68**	**36 (83.7%)**	**7(16.3%)**	

***p* –value 0.244**

Table 6: Distribution of CMV gB Glycoprotein Genotypes among HIV Positive Patients on HAART Based on Location of Residence

Location	No. Tested	gB1 no (%)	gB2 no(%)	95% CI
Urban	50	23(46.0%)	6 (12.0%)	0.948 , 10.414
Rural	18	13(72.2%)	1 (5.6%)	
n= 43	**68**	**36 (83.7%)**	**7(16.3%)**	

p* - value 0.021

Table 7: Distribution of CMV gB Glycoprotein Genotypes among HIV Patients on HAART in Bida Based on Marital Status.

Marital status	No. Tested	gB1 no (%)	gB2 no (%)	95% CI
Married	47	25(53.2%)	3 (6.4%)	0.837 ,10.247
Singles	21	11 (52.3%)	4 (23.5%)	
n= 43	**68**	**36 (83.7%)**	**7(16.3%)**	

p* - value 0.040

Table 8: Distribution of CMV gB Glycoprotein Genotypes among HIV Patients on HAART in Bida Based on Educational Status

Levels	No. Tested	gB1 no (%)	gB2 no (%)	95% CI
Primary	18	10(55.6%)	5 (27.8%)	0.139, 10.389
Secondary	28	16 (57.1%)	1 (3.6%)	
Tertiary	22	10 (45.5%)	1 (4.5%)	
n=43	**68**	**36 (83.7%)**	**7(16.3%)**	

p* - value 0.003

Table 9: $CD4^+$ Count Distribution of CMV gB Glycoprotein Genotypes among HIV Positive Patients on HAART in Bida

$CD4^+$ Count	No. Tested	gB1 no (%)	gB2 no (%)	95% CI
<200 cells/µl	17	9(52.9%)	2(11.8%)	400.8 , 533.2
>200 cells/µl	51	27 (52.9%)	5 (9.8%)	
n=43	**68**	**36(83.7%)**	**(16.3%)**	

***p* - value 0.426**

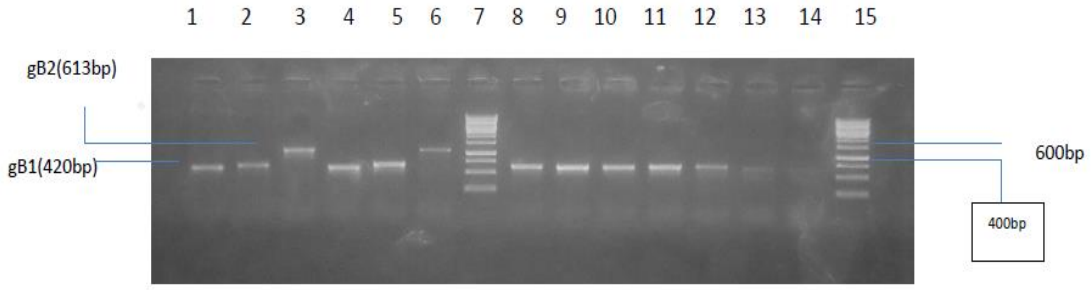

Fig 1: Agarose gel electrophoresis of the CMV gene subtypes from the patients. Lanes 3 and 6 represent the bands for the gB2 sub-types. Lanes 1, 2, 4, 5, 8-13 represent the bands for the gB1 CMV sub-types, 14 represents the negative control. Lane 15 represents a 200bp molecular ladder

DISCUSSION

The specific CMV IgM antibodies prevalence was found to be 77(19.8%), this prevalence reported in Bida was higher compared to reports among HIV patients in recent times in other developing countries, the prevalence of 11.1% was reported by Fowotade *et al.* 2015 (11) from HIV seropositive patients in Ilorin, while Ojide *et al.* 2013 (12) in Benin City reported 7.0%, *Musa et al.* 2014 (13) in Kano reported 13.0% and *Akimbami et al.* 2010 (14) in Lagos reported 6.6%. All reported a lower prevalence compared to our findings in Bida.

PCR in this study appeared to be superior in the detection of CMV infection in HIV, for the nested PCR analysis, 43 (63.2%) were positive out of the 68 genotyped for glycoprotein B of CMV among subjects. IgM ELISA was positive in 77 patients out of which IgG avidity showed that 12 were high avid and only 65 were really primary infection and these patients showing positive CMV IgM antibodies were however found to be negative by PCR.

Glycoprotein B of cytomegalovirus plays an important role in virus infectivity and correlation of gB type distribution in cytomegalovirus disease among immunocompromised patients with CMV infection (15). In the present study, from 43 subjects the gB genotype distribution by Multiplex nested PCR revealed that gB1 is the more prevalent strain in HIV subjects 36(83.7%) followed by gB2 7(16.3%) which is in agreement with reports from France, Brazil, Kuwait and Iran, (16,17 18 , 19). Thus gB3, gB4 and gB5 strains are not among the circulating strain among HIV patients in Bida and this is in contrast with (20) who reported a higher frequency of gB2 strains among AIDS patients with retinitis.

This study evaluated the molecular diversity of CMV genotypes in Bida. Our work reported only two strains gB1 and gB2 out of five glycoprotein B genotypes and there were no mixed infection in this study which was not in agreement with (21) who reported that two or more CMV strains are often found in AIDS patients.

This study revealed a higher distribution of glycoprotein B gB1 of CMV among female (55.1%) than male, while gB2 were more in females (15.1%). Gender distribution of CMV gB genotype among HIV patients is

not gender dependent among the subjects as it was deduced that gender have no role in gB genotype distribution. This study found no significant difference (p =0.767) between gender and glycoprotein B distribution among HIV patients.

This study observed a very high frequency of glycoprotein (gB1) distribution among age group 10-19 years old (100%) while (gB2) had and higher prevalence among age group 0-9 years (33.3%). Although there is paucity of literatures on the relationship between age and glycoprotein B distribution among HIV subjects, we observed that the younger age groups had more glycoprotein B distribution. Young children are known source of Human Cytomegalovirus infection in the population, they likely got infected through longitudinal transmission from other children and possibly indirectly through environmental contamination. Our findings revealed no significant association between age and CMV gB type distribution in HIV subjects in Bida (p = 0.244).

The distribution of gB1 genotype was higher among subjects from the rural settings (72.2%) compared to gB2 genotype which were more in subjects from the Urban area (12.0%). Rural setting is characterized by low socioeconomic level, ignorance about predisposing factors to CMV infection and the lack of basic hygiene practice contributed to the high distribution of glycoprotein B in rural settings in this study. Our result found a statistical difference between location of HIV subjects to gB glycoprotein genotypes distribution in Bida (p= 0.021).

The distribution of CMV gB genotypes among HIV subjects with marital status revealed that gB1 had higher prevalence of 53.2% among the married subjects while gB2 were higher among their single counterparts (23.5%).It was deduced from our findings that sexual activity plays a major role in CMV infection among sexually active adults. Polygamous practices among the married subjects are factor that greatly influence transmission of CMV infection by sexual relationship with infected spouse. This study found a statistical difference (p= 0.040) between marital status of HIV patients to gB glycoprotein genotypes distribution in Bida.

The distribution of gB1 genotypes was highest among secondary school education holders (57.1%) while gB2 are more among primary education holders (27.8%). Our findings showed that education plays an effective role as knowledge about ricks and prevention of CMV infection reduces incidence in subjects with higher education. This study found a statistical difference between educational status of HIV subjects to gB glycoprotein genotypes distribution in Bida (p= 0.003). There is an association between role of increased awareness of risk factors of acquiring CMV infection and gB glycoprotein distribution.

This study also determine the immune status of HIV seropositive patients to CMV with the distribution of the molecular diversity of gB glycoprotein B strains, the $CD4^+$count–related distribution of gB1 genotype revealed that both low and moderate $CD4^+$ count had the same prevalence of 52.9% in $CD4^+$ count < 200 cell/µl and< 200 cell/µl while gB2 genotype had high prevalence with low $CD4^+$ count < 200 cell/µl. There is no significant difference between $CD4^+$ count and the distribution of gB genotype among HIV in Bida.

Although, there was paucity of reports on CMV gB genotype distribution with socio demographic factors, this study found significant correlation in marital status, location and educational status, however age, gender, $CD4^+$ count are not statistically significant with gB genotype in Bida

CONCLUSION

This study revealed that there was no mixed infection in this study, all the primary infections are reactivation of CMV infection and also no incidence of reinfection of CMV was reported among HIV patients in this study. This work also evaluated the molecular diversity of CMV genotypes in Bida, more studies on CMV glycoprotein B genotype from Nigeria may help to determine the optimal strains for CMV vaccine development in Nigerian population however our work reported only two strains gB1 and gB2 out of five CMV gB glycoproteins.

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