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Detection of Cytomegalovirus Glycoprotein B Genotypes among Early and Late Stages of HIV Infected Sudanese Patients

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Abstract

The present research was conducted at the voluntary counseling and testing (VCT) center Khartoum, Sudan during 2015-2016. This is a cross-sectional study. A sum of 194 individuals were recruited to take part in this research (of them 96 were HIV patients in the first stage and 98 in the late stage of infection). Blood specimens were collected from each subject. The specimens were screened for cytomegalovirus CMV-antibodies (IgM and IgG) using ELISA (Acon Laboratories, Inc.), CMV-DNA was determined by Polymerase chain reaction PCR and CMV-gp (glycoprotein) B genotyping was performed by Restriction fragment length polymorphism (RFLP). Results of this investigation showed a significant prevalence of CMV infection. The results of HIV cases co-infected with CMV showed up that the disease is higher among females than males and among cases in late-stage more than those in the first stage. The age cohort 9-31 years, subjects on treatment and HIV cases with clinical manifestations displayed significant seroprevalence of CMV-IgM. CMV-gpB1 genotype was more frequent among sufferers in the late stage of disease, subjects with clinical manifestations and females. CMV-gpB2 and 3 genotypes were more frequent in males. We concluded that CMV and its related gpB genotypes may influence the diagnosis and outcome of AIDS cases.

Keywords: HIV; CMV infection; ELISA; AIDS

Introduction

Cytomegalovirus (CMV) is an omnipresent herpesvirus that often leads to symptomless or moderately symptomatic infections in immunocompetent subjects. Conversely, CMV infection in immunocompromised patients carries significant percentages of morbidity and fatality [1]. CMV disease is endemic across the globe. Infection with CMV is more common within the developing regions and among the general population who belong to the lower socioeconomic sections of society. The seroprevalence differs with a variety of epidemiologic factors, for example, age, topographical distribution, socioeconomic status, legal status, and equality [2,3]. In the vast majority with a completely functional immune system, the initial infection with CMV may provoke mild influenza like sickness and later the virus could stay dormant. A damaged immune system allows the reactivation of CMV. A synergistic impact weaken the immunological profile and could convert into a more rapid disease progression in HIV affected individuals [4]. The tumor necrosis factor (TNF-α) mediates stimulation of the host cell prompting the intranuclear accumulation of the nuclear factor- (NF-kB) and leads to the activation of the CMV DNA replication. This might justify the high prevalence of the CMV reactivation in HIV infection [1]. Through advanced AIDS, CMV can deliver debilitating end-organ disease (EOD) together with colitis, pneumonitis, and retinitis. Prior of the highly active antiretroviral therapy (HAART) era, a few investigations complied with that the rates of the CMV EOD among the cases with advanced HIV infection were around 40% or higher. With the presence of the drug cocktail (HAART), the frequency of the CMV EOD gets decreased [5,6]. CMV IgG antibodies are available in long-standing CMV seropositive individuals yet the clinical appearances of the CMV infection rarely introduce till the CD4 count falls under one hundred cells/cmm [7].

ELISA is the most accessible technique of measuring the antibodies to CMV. Measuring CMV IgG antibodies show that the individual had exposure to CMV. The CMV IgM antibody testing shows a recent or the reactivation of CMV infection [3]. Genotyping of CMV has been used to consider potential variations in pathogenicity of specific strains. but, few authors described a connection between specific CMV genotypes and the seriousness of the illness. Imperative, genotyping of CMV has enabled the discrimination of reactivation of latent virus from reinfection with new CMV strains in transplant patients, permitting a better definition of a donor to recipient transmission patterns. Congenital CMV infections arise from recurrent infections among pregnant ladies comprising each reactivation and reinfection. The discrimination of reactivation from reinfection could offer insight into the mother to fetus transmission pattern and then the possible associations with the outcome of congenital CMV infections. Genotyping of CMV has focused on envelope glycoproteins gB (UL55) and gH (UL75), that play a role in virus attachment and are major targets for neutralizing antibody response. The methods that most applied for genotyping of CMV are restriction fragment length polymorphism of PCR amplicon and nucleotide sequence analysis.

Materials and Methods

The research was conducted among HIV positive Sudanese patients at the VCT centers in Khartoum state. The ethical approval for the study was obtained from the ethical Board of The National Ribat University. Each participant was asked to sign a written consent to participate in

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the study. We formerly collected the data from each individual using interviewed questionnaire. The desired data include age, gender, residence, academic level and stage of disease.

Specimens collections

Five ml of venous blood were collected into EDTA vacutainers under aseptic manner from each patient under study. The specimens were centrifuged at 2000 rpm and the plasma separated into plain containers.

Each plasma sample was subdivided into two portions, one for serological screening of CMV antibodies using ELISA and the second portion of plasma for extraction of the DNA. The detection of the CMV DNA by nested PCR and genotyping by a restriction enzyme.

Detection of CMV antibodies

IgG and IgM antibodies to CMV were measured by ELISA (Acon Laboratories Inc., USA) according to the instructions of the manufacturer. Working wash buffer was prepared by diluting the concentrated wash buffer 1:25. Well A1 was left as blank well. Then 100 µl of calibrator 1 (yellow reagent) were added to wells B1 and C1, 100 µl of calibrator 2 and quantification of CMV gB and gH genotypes. However, they have been applied to plasma or other high volume samples. Likewise, deep sequencing-based strategies, sensitive in the detection of genotype mixtures with low ratios, needed a huge input of CMV genomes. Studies on genotyping of CMV in DBS are rare and are blocked by the small volume of dried blood available. (Blue reagent) in wells D1 and E1, 100 µl of calibrator 3 (blue reagent) in wells F1 and G1 and 100 µl of calibrator 4 (blue reagent) in wells H1 and A2. Starting from B2, 100 µl specimen diluent was added then 5 µl of the specimen was added to every corresponding well. The unused strip removed and frozen at 2-8°C. The plate was mixed then coated with the plate sealed and incubated at 37°C for a half-hour. We pulled out the plate sealer and again the micro-wells washed 5 times with 350 µl of operating wash buffer, thus turned to an inverted position on absorbent cotton. Hundred µl of the conjugate was added to every well except the blank well. The micro-well plate was closed with the plate fixed and incubated at 37°C for 30 minutes. The plate was then rinsed as above and we added 50 µl of substrate A and substrate to each well. The micro-well plate was covered with a plate fixed and incubated at 37°C for 10 minutes. The plate sealer was moved out and 50 µl of stop solution was added to every well, we read the absorbance at 450/630-700 nm at intervals 30 minutes.

Detection of CMV-DNA in the samples

The We performed PCR using CMV primers (Eurofins, Germany), as follows: gB1319 (5'TGGAACTGGAACGTTTGGC') and gB 1604 (5'GAAACGCGCGGCAATCGG-3') as an external primers (size 350 pb). In the second round we performed a nested PCR using gB 1319 (5' TGGAACTGGAACGTTTGGC3') and gB 1676 (5'TGACGCTGGTTTGGTTGAATG3') as an internal primer (size 296 pb). For the PCR, a reaction mixture was prepared to form 12.5 µL ready to use master mix 2x (Thermo Fisher Scientific, USA), 5.5 µL nuclease-free water (Thermo Fisher Scientific, USA) and 1 µL of every primer. To the current reaction mixture, 5 µL of extracted DNA was added, with a final reaction volume of 25 µL. The reaction mixture was incubated at 94°C for 3 min. Then the temperature was reduced to 80°C, once 2 U of Taq DNA polymerase was supplied and the mixture was submitted to 15 cycles of 60 sec at 94°C, 120 sec at 65°C, and 120 sec at 72°C, and to 30 cycles of 60 sec at 94°C, 90 sec at 55°C, 120 sec at 72°C, and to 3 min at 72°C. We utilized two microliters of those reaction products in the nested-PCR, included in a reaction mixture same to that mentioned above, except that the primers gB1319 and gB1676. PCR and nested-PCR products were undergoing to gel electrophoresis in a 2% agarose gel and the amplicon bands were visualized under UV light after ethidium bromide staining (UVP, USA). A hundred base pair (bp) marker (Amersham Pharmacia, USA) was used to find the size of bands. The PCR product of nested-PCR was furthermore used for genotyping CMV [8].

Genotyping cytomegalovirus of gp B by restriction enzyme

The PCR product of CMV obtained by nested PCR was submitted to an enzymatic cleavage for gB genotyping. For this situation, we added 10 µL of a mixture consisting of the amplicons to a microtube containing two µL of the appropriate buffer solution. The enzymes HinfI and RsaI (New England Biolabs, USA) (10 U, from each enzyme) were added to the solution, the volume is 20 µL. Following one hour of incubation at 37°C, we subjected the amplicon to gel electrophoresis at 100 V, for 45 min, then the digested products were seen in a 1% agar gel. This method allowed the differentiation of fragments of comparable weights (up to 10 bp), and the identification of the four described gB genotypes [9].

Statistical analysis

Analyses were accomplished operating a computer application for Statistics program (SPSS 13.0, SPSS Inc. Chicago, IL, USA). We analyzed the frequencies applying the Chi-Square test. The relationship between CMV positive and data of the questionnaire was tested by Pearson analysis. The P value <0.05 was considered statistically significant.

Results

We conducted the present study to determine the seroprevalence and gpB genotypes of CMV among HIV-infected patients that attended VCT centers in Khartoum State. A total of 194 HIV-infected patients were included in this study (101 males and 93 females). Ninety-eight of participants were in the first stage of illness and the remaining in the late stage. The mean age of participants was 49 years (+/- SD) ranged from 9 to 80 years. All subjects (100%) tested positive for CMV IgG antibodies but only 35 (18%) were CMV IgM positive. Seroprevalence of CMV IgM was higher (P value: 0.4) among patient in the late stage compared to those in the early stage (20.4% versus 15.6%). In terms of gender, the seroprevalence of CMV IgM was higher (P. value: 0.01) among females (24.8%) than males (10.8%). CMV IgM antibody detection was higher (P value: 0.05) among age group 9-30 years. Seroprevalence of CMV IgM was higher (P value: 0.04) among HIV patients with clinical symptoms (34.3% have pneumonia, 36.6% diarrhea, 20% retinitis, 60% HBV infection, 50% shingles and 25% Kaposi sarcoma). Out of the total number of subjects under study, we detected CMV-DNA in 15.5% (Figure 1).

In terms of gender, CMV-DNA positive cases were insignificantly (P value: 0.3) higher among females (16.8%) than males (14%). CMV-DNA positive cases were insignificantly (P value: 0.7) higher among age group 9-30 years. The rate of detection of CMV-DNA was significantly higher (P value: 0.04) among HIV-infected patients in the late stage (24.5%) than those in the early stage (6.3%) (Table 1).

Retinitis, encephalitis, pneumonia, diarrhea, and co-infection with hepatitis B were significantly (P value: 0.00) more frequent among CMV-DNA positive cases at the late stage compared to those at the early stage of HIV infection. Among the studied population, CMV-

DNA was higher among patients having retinitis (80%), followed by those who had encephalitis (50%), then pneumonia (28%), Kaposi sarcoma and shingles (25% each).

CMV-DNA detection was relatively higher (P value: 0.04) within subjects during therapy (18.8%). Out of the overall number of individuals under study (194), 35 samples were positive by ELISA whereas 30 samples positive by PCR. Out of 35 positives when using ELISA only 5 samples proved positive by PCR.

CMV-DNA was amplified by (PCR), and the amplicon was digested by a restriction enzyme, followed by electrophoresis. These restriction fragment length polymorphism (RFLP) analyses enabled the differentiation of 30 strains on 3 genotypes (Figure 2).

Yielded distinguishable digest profiles for groups 1-4. As described in Chou and Dennison, Marker 20, 50, 70, 100, 130, 200, 220, 250 bp. line 2, 5,11,14 (Glycoprotein B 3) 97 and 202 bp. Line 3, 4, 6, 7, 8, 9, 10, 12, 13 (Glycoprotein B 1) 36, 67 and 202 bp. Lane 15, 16,17 and 18 (Glycoprotein B 2) 100 and 202 bp.

Stage of HIV patients	Total examined	CMV-DNA positive using PCR	
		Frequency	%
Early	96	6	6
Late	98	24	24.5
Total	194	30	15.5

Table 1: Distribution of CMV-DNA positive HIV patients according to the stage of HIV illness.

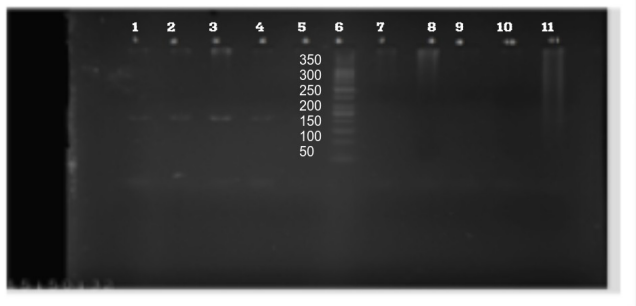

Figure 1: PCR result band size (296).

Lane 6 (50, 100, 150, 200, 250, 300, 350). Lane 1, 2, 3, 4 Positive Sample. Lane 7, 8, 9 and 10 Negative sample. Lane 11 PCR Negative control.

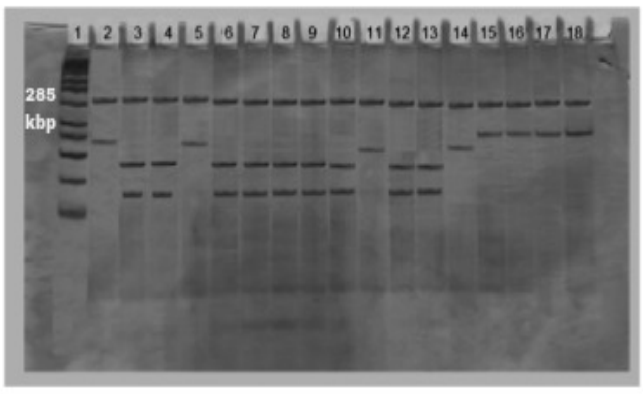

Figure 2: Restriction digests of amplified part of glycoprotein B (bases 1319-1604) and Enzymes HinfI.

The most frequent CMV gp B genotype was genotype 1 (53.3%) followed by genotypes 2 and 3 each (23.3%) while genotype 4 was not detected.

Genotype 1 was insignificantly (P value: 0.26) higher among late stages of HIV-infected patients. Genotype 2 was significantly higher among early stages of HIV-infected patients. Genotype 4 was neither detected in the patients in the first nor the late stages of HIV infection.

The frequency of CMV gp B genotypes among HIV positive Sudanese cases as per the stages of illness. Genotype 1 and 3 were significantly higher (P value: 0.00) among the age group (31-60) years, while genotype 2 was more frequent in age group (9-30) years. The frequency of genotype 1 was insignificantly (P value: 0.2) higher among patients with retinitis, diarrhea, and encephalitis. Genotype 2 more frequent among the asymptomatic patients while genotype 3 more in patients with diarrhea. The frequency of genotype 1 was insignificant (P value: 0.7) higher in females, while genotype 2 and 3 were increasingly frequent in males.

Discussion and Conclusion

In this study, participants (HIV-infected) tested positive for CMV-IgG antibodies. This is in agreement with studies done in Iran and Lagos in Nigeria [10,11]. A study done in Ghana revealed low (59.9%) a seroprevalence of CMV-IgG antibodies [12]. Out of the total number of HIV patients examined the seroprevalence of CMV IgM antibodies was 18%. A similar study by Leach et al. found 22% of the studied population positive for CMV-IgM antibodies [13]. Our result is lower than study done in Iraq which reported CMV-IgM seroprevalence to be 42.9% among AIDS patients [14]. The variation in seroprevalence of CMV IgM observed may be due to epidemiological and geographical differences. Seroprevalence of CMV IgM was insignificantly higher (P value: 0.4) among patient in the late stage compared to those in an early stage (20.4% versus 15.6%), that is like to the findings of study accomplished by Ojide et al. which showed the seroprevalence of CMV IgM to be significantly higher in patients in WHO clinical stages 3-4 (10.9%) than those in stages 1-2 (4.7%) [15]. There are reports on a synergistic interaction between HIV and CMV that is able to exacerbate the immunologic profile increased the progression to AIDS in HIV infected patient [4,16]. On the other hand tumor necrosis factor (TNF)-α stimulates host cells and accumulates intranuclear kB factor leading to activation of CMV DNA replication [16]. In the present study, females showed significantly higher (P value: 0.01) a seroprevalence of CMV IgM antibodies (24.8%) compared to males (10.8%). This result disagrees with a study by Fowotade et al. that showed seropositivity for anti-CMV IgG and IgM antibodies to be the same in both males and females [17]. Studies conducted in Brazil and Gambia revealed higher seroprevalence of CMV-IgM antibodies among females (94.7%, 87% respectively) which in agreement with our study [18,19]. The superiority of the female might be by the fact that they perhaps have of their contact with the infected children who are a significant sources of CMV infection [20]. Seroprevalence of CMV IgM among the HIV patients with clinical manifestations were 34.3% in pneumonia cases, 36.6% diarrhea, 20% retinitis, 50% shingles and 25% Kaposi sarcoma. Tumor necrosis factor (TNF)-α stimulates host cells and accumulates intranuclear kB factor leading to activation of CMV DNA replication [16]. In the present study, females showed significantly higher (P value: 0.01) seroprevalence of CMV IgM antibodies (24.8%) compared to males (10.8%). This result disagrees with a study by Fowotade et al. that showed seropositivity for anti-CMV IgG and IgM antibodies to be the same in both males and females [17]. Studies conducted in Brazil and Gambia revealed higher seroprevalence of CMV-IgM antibodies

among females (94.7%, 87% respectively) which in agreement with our study [18,19]. The superiority of the females might be clarified because they maybe have more contact with babies secretions such as saliva or urine of CMV infected children. This is expected as the immune status of patients in the former groups is likely to be more than the late groups, making them more susceptible to both re-infection and reactivation. In this study, the seroprevalence of CMV-IgM antibodies was significantly higher (P value: 0.05) among the age groups 9-31 years. PCR is the most accepted technique because of its accuracy. Out of the total number of HIV patients (194) studied, we found CMV-DNA in 30 subjects (15.5%). This rate of detection is in concordance with a study done in Brazil which reported a 16% [21].

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