Prevalence, Risk Factors, and Impact on Outcome of Cytomegalovirus Replication in Serum of Cambodian HIV-Infected Patients (2004–2007)

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Background: In developing countries, the study of cytomegalovirus (CMV) coinfection in HIV-infected patients remains neglected. Quantitative CMV polymerase chain reaction (PCR) is the gold standard diagnostic tool for analyzing serum CMV replication and for predicting CMV disease. We estimated the prevalence of replicating CMV in sera of newly diagnosed HIV-infected Cambodian patients and examined its impact on mortality.

Methods: This cohort study was based on 2 highly active antiretroviral therapy treatment programs in Cambodia between 2004 and 2007. Quantitative CMV PCR was performed on baseline serum samples of 377 HIV-infected patients.

Results: The prevalence of serum CMV DNA was 55.2% (150 of 272) in patients with $CD4^+$ count $<100/mm^3$. In multivariate analysis, hemoglobin <9 g/dL, $CD4^+$ count $<100/mm^3$, and Karnofsky index <50 were independently associated with positive serum CMV DNA at baseline. During a 3-year follow-up period, CMV viral load ≥ 3.1 $\log_{10}$ copies per milliliter was significantly associated with death independently of $CD4^+$ count, other opportunistic infections, and highly active antiretroviral therapy.

Conclusions: As in industrialized countries, serum CMV replication is highly prevalent among HIV-infected Cambodian patients and is associated with increased mortality. This underscores the importance of diagnostic CMV infection by PCR in sera of HIV-infected patients with $CD4^+$ count $<100/mm^3$ and treating this opportunistic infection to reduce its associated mortality.

Key Words: Cambodia, CMV, HIV, mortality, PCR, prevalence

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INTRODUCTION

Cytomegalovirus (CMV) disease is an opportunistic infection in HIV-infected patients, resulting from reactivation of latent infection in patients with advanced immunosuppression.

In resource-limited settings, the diagnosis and the treatment of CMV disease are generally not available and CMV disease remains neglected. In previous studies conducted in industrialized countries, detectable CMV DNA by polymerase chain reaction (PCR) in plasma[1,2] or in whole blood[3] was an independent predictor of death even after adjusting for HIV RNA level or CD4 cell count. In Spanish patients with advanced HIV infection, CMV viremia was even the strongest predictor of death.[4]

In this study, we estimated the prevalence of serum CMV DNA in a cohort of 377 newly diagnosed HIV-infected Cambodian patients. Serum CMV DNA was detected by the use of a real-time qualitative and quantitative PCR technique. Relations between CMV PCR qualitative and quantitative results, clinical data, and patient survival were analyzed in this cohort.

PATIENTS AND METHODS

Cohort Intake

This cohort was built within the highly active antiretroviral therapy (HAART) access program at Prea Bat Norodom Sihanouk Hospital [program supported by Médecins Sans Frontières (MSF), France] and Calmette Hospital

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(program supported by Médecins Du Monde, France), Phnom Penh, Cambodia. It was based on the follow-up of HIV-infected patients initially enrolled in a study of opportunistic infections and more specifically assessing the clinical utility of serum cryptococcal polysaccharide (CPS) for the diagnosis of cryptococcal infection.[5] Between April and November 2004, all HAART-naive HIV-infected adult patients seen for the first time as inpatients or outpatients were invited to participate in the study. After signing an informed consent, patients answered a questionnaire on sociodemographic characteristics and medical history and were subjected to an in-depth clinical examination. Investigations included a cerebrospinal fluid (CSF) examination for patients with symptoms suggestive of meningoencephalitis and a chest x-ray for patients with respiratory symptoms. A blood sample was taken for CD4 cell count (CD4 count), serum CPS detection, and other analyses (see laboratory examinations). Details of the study have been reported elsewhere.[5]

Cohort Follow-Up

In 2007, after investigations showing high prevalence of CMV retinitis among patients with CD4 counts $<50/mm^3$ in several MSF HAART programs,[15] we decided to perform a retrospective analysis of the serum samples collected in 2004 in Phnom Penh for the detection of CMV DNA. We also reviewed the databases of the 2 treatment programs to determine the outcome (dead/alive/lost to follow-up) of the 377 HAART-naive HIV-infected adults screened in the initial study. An active procedure was conducted between February and April 2007 to trace patients lost to follow-up. At the time of assessment, 9.4% (35 of 377) patients could not be traced because of lack of proper identification. Of the 342 remaining patients, 100 (29.2%) were dead, 119 (34.8%) were still alive, and 123 (36.0%) were lost to follow-up (ie, were late by more than 3 months for their last scheduled visit).

Laboratory Analysis

During the 2004 survey, all patients provided a 10-mL blood sample that was transported the same day to the Institut Pasteur of Cambodia for CD4 count (FACScount; Becton Dickinson, Franklin Lakes, NJ), serum CPS detection (CALAS; Meridian Bioscience Europe, Nice, France), and storage at −20°C. Cryptococcal infection was defined by a positive CPS agglutination test (in serum or CSF) or positive *Cryptococcus neoformans* culture (in blood, CSF or urine) or positive india ink direct examination of CSF as described previously.[6]

In 2007, stored sera were used for CMV DNA detection by PCR. DNA was extracted from 200 μL of serum with the MagNA Pure Compact (Roche, Osterode am Herz, Germany) following the MagNA Pure Compact Nucleic Acid Isolation Kit I protocol. Extracted DNA was eluted in 100 μL of the elution buffer provided by the manufacturer. The CMV real-time PCR was conducted according to the protocol used since 2001 in the Virology Laboratory of Necker University Hospital (Paris, France). This test is an in-house CMV real-time PCR assay detecting a highly conserved region (74 base pairs) of the CMV UL123.[7] PCR was performed in a 25 μL volume containing 12.5 μL Platinum qPCR superMix-UDG (Cat. No. 11730-025; Invitrogen, Cergy Pontoise, France), 400 nM of each primer, 200 nM of TaqMan probe (Applied Biosystem, Courtaboeuf, France), and 5 μL of extracted DNA. Reactions were performed on the iQ5 Real-Time PCR Detection System (Biorad, Hercules, CA). Quantification was done with serial dilutions (10^5 to 10 copies/PCR reaction) of a prequantified plasmid containing the amplicon. Positive and negative controls were added to each series. The sensitivity of the CMV PCR test was 500 copies per milliliter (2.7 $\log_{10}$ copies/mL).

This initial study and its 2007 extension were approved by the National Ethics Committee for Health Research of the Cambodia Ministry of Health and by the Biomedical Research Committee of Pasteur Institute.

Statistical Analyses

Continuous variables were presented with their median and interquartile range (IQR). Variables were compared across groups using the Mann–Whitney *U* test for continuous variables and the χ^2 or Fischer exact test for categorical variables. CD4 count categories were chosen based on thresholds reflecting known levels of immunosuppression (≤50, 51–100, 101–200, and $>200/mm^3$), whereas body mass index and hemoglobin categories were based on quartiles.

Factors associated with serum CMV DNA detection were identified using logistic regression. Variables with *P* values <0.25 in univariate analysis were tested simultaneously in the multivariate model and removed after a backward stepwise procedure until all variables left in the model had *P* values <0.05. For multiple categorical variables, significance was tested using a maximum likelihood procedure.

Kaplan–Meier curves were used to estimate the survival probability of patients according to their CMV DNA status at inclusion in the cohort. A Cox model was used to estimate hazard ratios associated with the mortality during the 3-year follow-up. All variables introduced in the model were baseline characteristics, except for HAART, which was treated as a time-dependent variable (ie, patients contributed person-time to the treated category only while under HAART). Patients lost to follow-up were censored at the time they were lost to follow-up.

For all analyses, statistical significance was defined as $P < 0.05$. Statistical analyses were performed using SAS 8.02 (SAS Institute, Inc, Cary, NC).

RESULTS

Characteristics of the Patients

The baseline characteristics of the study population (N = 377) are summarized in Table 1. Approximately half of the patients were male (50.9%) and were recruited through inpatient (45.9%) or outpatient (54.1%) department. Median (IQR) age was 35 (31–40) years. Patients were severely immunosuppressed, as shown by low median (IQR) Karnofsky index [70 (60–80)], body mass index [17.3 (15.6–19.3) kg/m^2], and CD4 count [30 (9–120)/mm^3]. Most patients presented clinical symptoms, 38.9% of them having temperature ≥38.0°C, 60.2% complaining of headache, and 52.1% diarrhea.

TABLE 1. Characteristics of 377 HIV-Infected Patients According to Results of CMV PCR

	Total (N = 377), n (%)	Positive CMV PCR (n = 160), n (%)	OR (95% CI)	*P*
Type of service*				
Inpatients	172 (45.9)	106 (61.6)	4.6 (3.0 to 7.1)	10^{-4}
Outpatients	204 (54.1)	53 (26.0)	1	
Age class in years				
<29	67 (17.8)	28 (41.8)	1.2 (0.6 to 2.3)	NS
29–39	211 (56.0)	95 (45.0)	1.4 (0.8 to 2.2)	
>39	99 (26.2)	37 (37.4)	1	
Sex				
Male	192 (50.9)	96 (50.0)	1.9 (1.3 to 2.9)	0.003
Female	185 (49.1)	64 (34.6)	1	
Karnofsky index†				
≤50	66 (17.6)	47 (71.2)	4.4 (2.5 to 7.9)	$<10^{-4}$
>50	309 (82.4)	111 (35.9)	1	
Body mass index				
≤15.6	93 (24.7)	56 (60.2)	5.0 (2.6 to 9.3)	$<10^{-4}$
15.6–17.3	94 (25.0)	45 (47.9)	3.0 (1.6 to 5.6)	
17.3–19.3	95 (25.3)	36 (37.9)	2.0 (1.1 to 3.8)	
>19.3	94 (25.0)	22 (23.4)	1	
Clinical signs				
Temperature (°C)				
≥38.0	146 (38.9)	79 (54.1)	2.2 (1.5 to 3.4)	0.0002
<38.0	229 (61.1)	79 (34.5)	1	
Diarrhea				
Presence	196 (52.1)	83 (42.4)	1.0 (0.7 to 1.5)	NS
Absence	180 (47.9)	77 (42.8)	1	
Headache				
Presence	227 (60.2)	79 (34.8)	0.5 (0.3 to 0.7)	0.0002
Absence	150 (39.8)	81 (54.0)	1	
Neck stiffness				
Presence	32 (8.5)	17 (53.1)	1.6 (0.8 to 3.3)	NS
Absence	345 (91.5)	143 (41.4)	1	
Severe neurological signs				
Presence	18 (4.8)	10 (55.6)	1.7 (0.7 to 4.5)	NS
Absence	359 (95.2)	150 (41.8)	1	
Associated opportunistic infections				
Cryptococcal infection				
Presence	58 (15.4)	31 (53.5)	1.7 (1.0 to 3.0)	0.07
Absence	319 (84.6)	129 (40.4)	1	
Tuberculosis				
Pulmonary	32 (8.5)	18 (56.3)	2.2 (1.1 to 4.6)	0.001
Extrapulmonary	70 (18.6)	41 (58.6)	2.4 (1.4 to 4.2)	
Absence	275 (72.9)	101 (36.7)	1	
Pneumocystosis				
Presence	8 (2.1)	6 (75.0)	4.3 (0.7 to 31.1)	0.06
Absence	369 (87.9)	152 (41.2)	—	
Blood tests				
CD4 cell count/mm^3				
≤50	224 (59.4)	133 (59.4)	29.2 (8.9 to 96.1)	$<10^{-4}$
51–100	48 (12.7)	17 (35.4)	11.0 (3.0 to 40.3)	
101–200	42 (11.2)	7 (16.7)	4.0 (1.0 to 16.5)	
>200	63 (16.7)	3 (4.8)	1	

TABLE 1. (*continued*) Characteristics of 377 HIV-Infected Patients According to Results of CMV PCR

	Total (N = 377), n (%)	Positive CMV PCR (n = 160), n (%)	OR (95% CI)	*P*
Hemoglobin (g/dL)‡				
≤9.0	115 (31.6)	74 (64.3)	5.7 (2.9 to 11.2)	$<10^{-4}$
9.0–10.6	68 (18.7)	30 (44.1)	3.9 (2.2 to 6.7)	
10.6–12.0	110 (30.2)	35 (31.8)	2.3 (1.2 to 4.2)	
>12.0	71 (19.5)	17 (23.9)	—	

Percentage in the positive CMV PCR column is calculated for each line.
NS, not significant; OR, odds ratio.
*n = 376.
†n = 375.
‡n = 364.

Presence of Serum CMV DNA

CMV DNA was present in the serum of 42.4% (160 of 377) patients. As shown in Table 1, prevalence was higher in febrile patients when compared with others (54.1% versus 34.5%, $P = 0.0002$), in patients with opportunistic infections (tuberculosis, cryptococcal infection, and pneumocystosis with borderline significance for the latter two), and in patients with higher levels of immunodepression. In particular, CMV DNA was present in 59.4% (224 of 377) patients with CD4 count ≤50/mm^3 and only in 4.8% in patients with CD4 count >200/mm^3. Of interest, CMV DNA presence was also strongly associated with anemia, that is, being higher among patients with lower hemoglobin levels. This association remained in multivariate analysis, where Karnofsky index, CD4 count, and hemoglobin level were all independently associated with positive CMV DNA detection (Table 2).

The median (IQR) CMV viral load was 3.6 (3.1–4.2) $\log_{10}$ copies per milliliter. Median (IQR) CMV viral load was higher in patients with CD4 count ≤50/mm^3 when compared with >50/mm^3: 3.7 (3.1–4.3) $\log_{10}$ copies per milliliter versus 3.4 (2.9–3.7) $\log_{10}$ copies per milliliter, respectively (Wilcoxon test, $P = 0.01$).

TABLE 2. Multivariate Analysis of Risk Factors Associated With Positive CMV PCR in 364 HIV-Infected Patients

	Adjusted Odds Ratio (95% CI)	*P*
Karnofsky index		
≤50	2.6 (1.4 to 4.9)	0.003
>50	1	
CD4 cell count/mm^3		
≤50	17.9 (5.3 to 60.2)	$<10^{-4}$
51–100	7.0 (1.8 to 27.0)	
101–200	2.8 (0.6 to 12.3)	
>200	1	
Hemoglobin (g/dL)*		
≤9	3.8 (1.8 to 7.9)	0.0006
9–10.6	2.0 (0.9 to 4.5)	
10.6–12.0	1.6 (0.7 to 3.5)	
>12.0	1	

*Missing value of hemoglobin level for 13 patients.

Mortality

Figure 1 displays the survival curves according to serum CMV DNA presence. Patients with positive serum CMV DNA at baseline had higher mortality when compared with others (log-rank test, $P < 10^{-4}$). For patients with a negative CMV DNA, the Kaplan–Meier death rate was 9.6% at 6 months, 17.3% at 12 months, and 25.3% at 24 months. For patients with positive serum CMV DNA, these rates rose to 24.8%, 35.6%, and 53.62%, respectively. The hazard ratio [95% confidence interval (CI)] associated with serum CMV DNA was 2.7 (1.8 to 4.0, $P < 10^{-4}$). In an analysis restricted to the group of patients with CD4 count ≤50/mm^3, the presence of serum CMV DNA was still associated with death [log-rank test, $P = 0.005$; hazard ratio (95% CI), 2.0 (1.2 to 3.4) $P = 0.006$].

In multivariate analysis (Table 3), factors associated with death were low baseline CD4 count, cryptococcal infection at admission, and high baseline CMV DNA load. The CMV DNA load was associated with increased risk of death only for values higher than 3.1 log copies per milliliter. Compared with patients with undetectable CMV DNA load, the hazard ratio (95% CI) of death was 2.0 (1.1 to 3.8) for those with viral load between 3.1 and 3.5 and was 3.6 (2.0 to 6.8) for those with viral load higher ≥4.2 log copies per milliliter.

DISCUSSION

This is the first study evaluating the burden of CMV infection by real-time PCR in HIV-infected patients in a developing country. The prevalence of serum CMV DNA was very high, reaching 59.4% (133 of 224) among patients with CD4 count ≤50/mm^3 and of the same magnitude (28%–45%) as that reported in industrialized countries before the era of HAART for patients with <100 CD4/mm^3 count.[1,8] This high prevalence is in accordance with the 93.3% prevalence of anti-CMV total antibodies found in the sera of 359 blood donors in a neighboring country, Thailand.[9] This study being retrospective, routine retinal examinations were not performed and the prevalence of CMV-related disease in our population was not documented. However, a positive CMV PCR result has been reported to be a strong predictor of CMV disease in different studies conducted before and after HAART.[2,8,10,11]

The present study showed that in a developing country, a positive CMV PCR in serum is a strong predictor of death for

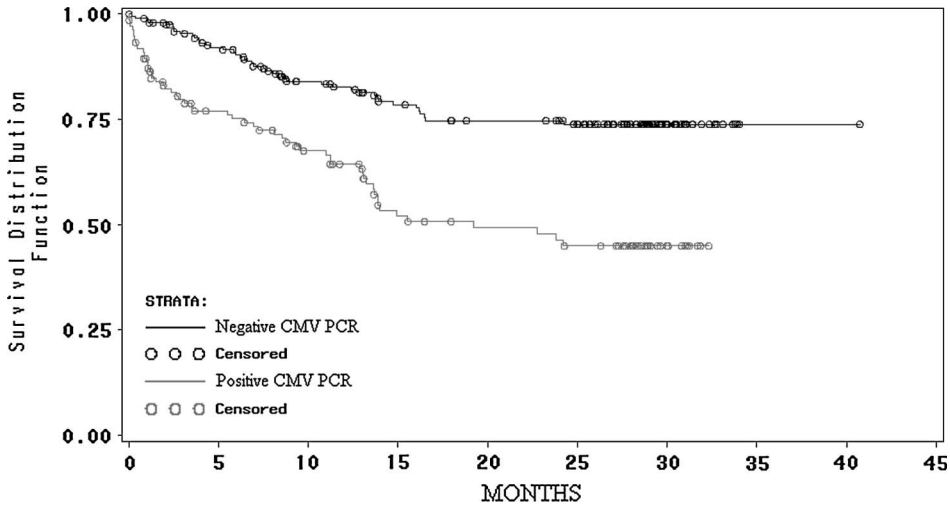

FIGURE 1. Survival of 342 patients according to serum CMV PCR at baseline. Among 377 patients, 35 patients could not be traced. Log-rank test $<10^{-4}$.

HIV-infected patients even after adjusting for CD4 cell count, HAART, and cryptococcal infection, highly prevalent in this area.

This study adds to existing knowledge by showing that increased mortality was observed only above a specific threshold of CMV viral load, in this case 3.1 $\log_{10}$ copies per milliliter (Table 3). In a previous study, it was reported that each $\log_{10}$ increase in baseline CMV DNA load in plasma was associated with a 2.2-fold increase in mortality.[1] Here, the identification of a threshold value could help to detect patients at higher risk of mortality.

TABLE 3. Univariate and Multivariate Analysis of the Mortality Risk Factors (Cox Model) in HIV-Infected Patients (n = 340)

	Hazard Ratio (95% CI)	
	Univariate	**Multivariate**
HAART		
Presence	0.55 (0.29 to 1.05)	0.36 (0.18 to 0.71)
Absence	1	1
$CD4^+$count/mm^3*		
≤50	2.3 (1.5 to 3.5)	3.0 (1.3 to 7.1)
51–200	0.8 (0.5 to 1.2)	2.6 (1.1 to 6.2)
>200	1	1
Cryptococcal infection		
Presence	2.3 (1.4 to 3.7)	2.1 (1.3 to 3.5)
Absence	1	1
CMV PCR ($\log_{10}$ copies/mL)*		
0–2.6	1	1
2.7–3.0	1.2 (0.6 to 2.1)	1.4 (0.7 to 2.8)
3.1–3.5	1.7 (1.0 to 2.9)	2.0 (1.1 to 3.8)
3.6–4.1	1.8 (1.1 to 3.3)	2.3 (1.2 to 4.4)
≥4.2	3.4 (2.0 to 5.7)	3.6 (2.0 to 6.8)

Lost to follow-up patients have been censored at that point in time. For 2 patients, date of HAART initiation was not available.
*$P < 0.0001$.

As shown elsewhere,[12] low hemoglobin levels were strongly associated with a positive CMV PCR [odds ratio (95% CI) = 3.8 (1.8 to 7.9)] when comparing patients with hemoglobin ≤9 to >12 g/dL. It may be speculated that anemia could simply be a marker of advanced immunosuppression and thus of higher risk of CMV viremia. However, the association remained in multivariate analysis, independently of CD4 cell count, thereby suggesting that anemia may indeed be a consequence of CMV viremia, a fact which has been documented earlier either as inhibition of erythropoiesis[13] or as hemolytic anemia.[14]

We agree with Heiden et al[15] that CMV-related pathogenesis is a neglected component of the AIDS epidemic in developing countries. Until specific diagnosis and treatment of CMV disease are available, some of the newly diagnosed HIV-infected Cambodian patients will continue to die of CMV infection before starting HAART and others will rapidly develop CMV retinitis, which might lead to blindness either before starting HAART or as a result of immune recovery uveitis under HAART treatment.[16–18] In resource-limited countries like Cambodia, it should be a priority to facilitate and improve the diagnosis of retinitis by indirect ophtalmoscopy in all HIV-infected patients with CD4 count $<100/mm^3$. In laboratories equipped with PCR facilities, detection of CMV DNA may also be of value to detect patients at high risk of CMV disease. Finally, intravenous infusion of ganciclovir or oral valganciclovir associated with intraocular injection of ganciclovir should be made available for treatment of CMV disease in HIV-infected patients of the developing world.[15,19,20]

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results for CMV real-time PCR. Gilles Guerrier collected the data outcome of the patients 3 years after their enrollment in the study done in 2004 and participated in the statistical analysis. Laurent Ferradini, Jean-Philippe Dousset, and Hak Chanroeun followed the patients in the program MSF and Médecins Du Monde and Esther programs. Philippe Jean Guerin and Suna Balkan contributed to the study design and the epidemiological analysis. Romain Micol, Philippe Buchy, Arnaud Fontanet, Olivier Lortholary, Christine Rouzioux, and Marianne Leruez-Ville were responsible for study design, data validation, data analysis, data interpretation, and article preparation. All authors have seen and approved the final version of the article.

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