Prospective Study for CMV Detection in Cancer Patients: Comparison between PCR, Antigenemia, and Serological Assays

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Abstract: ***Background.*** Human cytomegalovirus (HCMV) is a persistent pathogen, can cause life threatening infection in immunocompromised patients. Therefore, sensitive and reliable diagnostic test is essential for choice line of antiviral therapy and patients' management. ***Methods*:** HCMV detection was investigated by serological assay, PCR assay in both WBC's and plasma samples, antigenemia assay in PMBLs in 32 leukemia patients, 30 bladder cancer patients and 27 apparently healthy controls. ***Results:*** HCMV IgG was detected in 100% of our cancer patients. High HCMV IgG Ab titer was found to be less frequent in leukemia patients than solid tumor patients. In other words, low HCMV Ab titer was most common in leukemia patients. Antigenemia assay was positive in 8/32 (25%) of leukemia patients but viremia was positive in only 2/32 (6%) of leukemia patients. High level of antigenemia was associated with development of severe HCMV disease in leukemia patients. **Conclusion:** Antigenemia test is a reliable diagnostic tool in diagnosing active HCMV infection and progression. Therefore it can be helpful in guiding therapy in leukemia patients.

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1. Introduction

Human cytomegalovirus (HCMV) is a widespread pathogen responsible for asymptomatic and persistent infections in healthy individuals. In developing countries where poor hygiene and overcrowdings, children acquire infection early and seroprevalence approaches 100% by early adulthood. HCMV like all herpes viruses establishes latency and reactivates under immunocompromised conditions like those with human immunodeficiency virus (HIV) infection, patients following allogeneic stem cell transplantation (SCT) or organ transplantation and patients with hematological malignancies [(1,2)]. In immunocompromised hosts, CMV infection can causes various life- threatening diseases like; pneumonitis, hepatitis, gastrointestinal diseases, retinitis, etc. Also, it can increase incidence of other opportunistic infections and decrease survival of patients. In addition, they have reported that mortality rate due to CMV pneumonia was higher among lymphopenic patients highlights the important role of lymphocytes in controlling CMV infection [(3)].

Since, the choice of suitable strategy of antiviral line of therapy to prevent development of CMV infections and significant disease management in immunocompromised hosts depends on sensitive, accurate and reliable diagnostic tests, several reports demonstrated relevant methods are preferred for diagnosis of HCMV infection such as; detection of pp65 antigen from peripheral blood leukocytes (antigenemia assay) and CMV DNA [(4)]. However, such reports presented many discrepancies between these methods. Also their results were not obtained from patients with hematological malignancies. Therefore, the present study aimed at comparing results of different methods that can be helpful in diagnosing active HCMV infection. Consequently, accurate methods for determination of CMV infection will help in management of CMV infection and disease, and preventing development of drug resistant CMV mutant strains and in turn advanced line of treatment.

2. Patients and Methods

Patients

This study was conducted on 89 adult participants divided into three groups. The first group comprised 32 leukemia patients (12ALL, 12 AML, 6 CML, and 2 CLL) FAB classification was applied to ALL patients categorizing them into L1, L2 and L3 according to bone marrow morphology [(5)]. The second group comprised 30 patients with bladder cancer diagnosed and treated at the Medical Oncology Department, National Cancer Institute (NCI), Cairo University between September 2010 and October 2011. The third group 27 apparently healthy adults, as normal controls. The Institutional

Review board (IRB) of the NCI approved the protocol. Informed written consent was obtained from guardians of all adults enrolled in the study.

Disease extent and staging were established after a detailed history and physical assessment. Other baseline and prognostic investigations were carried out; complete blood count (CBC), liver and renal function tests. Individuals among bladder cancer and normal groups were subjected only to routine hematological investigations.

Specimen collection

Blood specimens were collected into tubes with EDTA (Ethylenediaminetetraacetic acid) anticoagulant, left clot for 15 min. at 37°c followed by refrigeration at 2-8°c for another 30 min. Blood was then centrifuged at 400xg for 10 min. Serum was collected and stored in small aliquots at -20°c until examined. The plasma was separated and centrifuged at 800xg for 10min. Samples of plasma were collected in EDTA coated tube, left at room temperature for 1-2 hours for sedimentation. The upper cloudy layer was transferred into clean sterile tube, centrifuged at 800xg for 10 min. and the supernatant fluid (plasma) was stored at -20°c. The pellet was suspended in 0.8% ammonium chloride at 4°c for 3 min. to lyse residual RBCs, followed by rising in PBS. This last step was repeated and the sedimented peripheral blood leukocytes were harvested and stored at -20°c for DNA extraction, and CMV-DNA amplification.

Serological detection of HCMV-IgM by ELISA

Analysis of serum samples for IgM antibodies to CMV was performed using CMV EIAGEN-M kit (clone systems, Italy). Assay procedure was done according to manufacturer's instructions kit. The concentration of antibodies in the sample was estimated using a calibration curve. The unknown samples whose absorbance values were greater than the cut-off values were considered reactive to IgM to CMV. The unknown samples whose absorbance values were less than cut-off values were considered non-reactive.

Serological detection of HCMV-IgG by ELISA

Analysis of serum samples for IgG antibodies to CMV was performed using CMV ELISA kit (Diamedix-Florida) Assay procedure was done according to manufacturer's instructions kit. The CMV value of the negative control should be less than 18. The CMV value of the positive control should be greater than 23. The unknown samples whose CMV values were greater than 23 were considered positive to CMV-IgG.

Determination and quantitation of HCMV-antigenemia assay:

Antigenemia assay was performed using immunostaining technique. The quantitative detection of HCMV antigen in polymorphonuclear leukocytes (PMNLs) cytospins were stained using mouse monoclonal antibody (supersensitive antibody, Biogenex, USA) directed against pp65 kD early nonstructural antigen. DAB kits were used for visualization of immunostaining technique [6].

Positive and negative control slides were included in each staining run. Controls and specimen slides were examined under light microscope using 400x magnification. Positive slide demonstrate CMV-Ag+ leukocytes characterized by a distinct brown perinuclear or nuclear staining. The +ve cells were counted and recorded.

Antigenemia is expressed by CMV antigen positive PMNL in aliquot of 200,000 cells. Low level of antigenemia expressed as presence of fewer than 10 positive cells/200,000cells. High level of antigenemia expressed as presence of more than 10 positive cells/200,000cells.

Molecular detection

1. Nucleic acid extraction:

Viral DNA was extracted from PMNLs and plasma of both leukemia and bladder cancer patients according to Zipeto *et al.* [7].

2. Amplification of HCMV- DNA

HCMV DNA was selectively amplified by PCR using sequence-specific primers form (shuttle Bioteck, Italy). This primer set shown to be specific for CMV and not to amplify DNA from other members of the herpesvirus family. 10 µl of DNA extract was subjected to DNA amplification in 90 µl of reaction buffer containing 10 mM Tris-HCL (pH 8.3), 1.5 mM $MgCl_2$, 20 nmol of each deoxynucleotide triphosphate, 50 pmol of each primer, and 2.2 units of Amplitaq (Perkin-Elmer Cetus, Norwalk, CT). The reaction was performed in a DNA thermal cycler (Perkin Elmer- Cetus): (i) an initial denaturation step of 5 min at 95°C; (ii) 50 cycles, with 1 cycle consisting of 40 sec of denaturation at 95°C, 50s of annealing at 62°C, and 40sec of extension at 72°C; and (iii) After the cycling program, the samples were incubated for 10 min. at 72°C. PCR was performed in a Perkin Elmer-Cetus thermo cycler [8].

3- Detection of Amplified products by agarose gel electrophoresis:

15 µl of PCR product was subjected to electrophoresis on a 2% agarose gel (Sigma) in Tris-Acetate buffer (TAE) pH 7.5-7.8, stained with 0.5ug /ml ethidium bromide and examined under UV transillumination and photographed, the expected CMV PCR band was at the level of 326 bp fragment.

Statistical methods

SPSS version 17 statistical software package for windows was used for analyzing the correlation between different immunological parameters in the

study groups. Mean/Median were used for quantitative data description. Mann Whitney test was used for comparison of 2 independent groups and non-parametric ANOVA was used for >2 independent groups. Chi square (χ^2) test or Fisher exact test were used for comparison of proportions. *P* value is significant at 0.05 levels.

3. Results

Demographic features of the studied groups

Distribution of age and sex in the 89 studied individuals was shown in Table 1. Preponderance of males was observed among bladder cancer patients and apparently healthy control group.

Detection of HCMV Antibody titer in the Studied groups.

All the 32 leukemic and the 30 bladder cancer patients had IgG Ab titer (100%) in comparison to 96% of the control group (Table 2). Three titer results were seen; low titer <80, intermediate titer 80-160 and high titer >160 (more than two fold of low titer (80EA) was taken as an indication of viral activity) among studied groups.

Regarding antibody titer and its distribution, our results showed that number of leukemia patients with high HCMV Ab titer was 9/32 (28%) compared with 18/30 (60%) and 23/27 (85.2%) in both bladder cancer patients and control groups. Were as number of leukemia patients with low HCMV Ab titer was 5/32 (15.6%) compared with 2/30 (6.7%) and 2/27 (7.4%) in both bladder cancer patients and control group, (Table 2). On the other hand, HCMV-Ig M antibodies were negative for all cases and control group.

Regarding HCMV antibody titer in the different age groups, low and intermediate HCMV Ab titer (<80, 80-160EA) were found to be more common (91.7%) in young leukemia patients (<25Y) compared to a frequency of (12.5%) in the old age group (>50 Y). Were as, high HCMV Ab titer (>160EA) was detected in most of old age leukemia patients (>50Y) (87.5%), compared with (8.3%) of the young age group showing a high statistical significant difference (P=<0.05), while no difference was found in the antibody titer among cancer bladder patients or healthy controls as regards age groups (P=>0.05) (Table 3).

Detection of HCMV DNA and pp-65 antigenemia in leukemia patients

Of the 32 leukemic patients 8(25%) were found to be positive by PCR, 6 (19%) in leukocytes and two cases (6%) were viremic as detected in plasma by PCR assay. Results are shown in Figure 2. Regarding antigenemia results, 8 out of 32 leukemic patients (25%) were positive for pp65 in their PMNLs. Quantitative results were available for those 8 positive patients: 4 (12.5%) patients had fewer than 10 positive cells/200,000 cells examined (low level) and 4 (12.5%) patients had 10 or more positive cells/200,000 cells examined (high level) (Table 2). Moreover, when comparing HCMV results obtained by PCR with that obtained by antigenemia assay. Our results showed that out of 8 positive cases for antigenemia assay, 2 (25%) patients showed negative results by PCR assay (Figure 2).

In addition, out of 8 positive cases for antigenemia assay, 3 (37.5%) cases had high Ab titer (>160EA) and only one case (12.5%) had low Ab titer (<80EA).

Correlation of HCMV Ab titer and antigenemia with the severity of HCMV disease

Leukemic patients were divided into 2 groups according to the severity of CMV disease into: Patients with mild HCMV disease (clinical score <7), and those with severe HCMV disease (clinical score >=7). Results showed that there was no difference in distribution of low and high Ab titer among patients with mild or severe HCMV disease. But the number of leukemia patients with high antigenemia level (>10Ag+ve/2x10^5 cells) were more among leukemic patients with severe HCMV disease (Figure 3).

Table 1.Demographic features of the studied groups

Group		Age (years)		Sex		
		Median	Range	Male	Female	M:F Ratio
Leukemia	32	40	18-80	17	15	1.1:1
Bladder cancer	30	55	24-72	21	9	2.3:1
Controls	27	34	20-65	21	6	3.5:1
Total	**89**			**59**	**30**	**2:1**

Table 2 Detection of HCMV infection by 3 different methods in the studied groups

Parameter	Leukemia group N=32		Bladder cancer group N=30		Normal control group N=27		*P* value
	No of +ve cases	%	No of +ve cases	%	No of +ve cases	%	
-ELISA (IgG) assay:	**32**	**100**	**30**	**100**	**26**	**96.3**	**<0.05****
Low titer (<80 EA*)	5	15.6	2	6.7	2	7.4	
Intermediate titer (80-160 EA)	18	56.4	10	33.3	1	3.7	
High titer (>160 EA)	9	28	18	60	23	85.2	
-PP-65 antigenimia assay:	**8**	**25**	**1**	**3**	**0**	**0**	**<0.05****
Low level (<10Ag+/2x10^{5}cells)	4	12.5	0	0	0	0	
High level (>10Ag+/2x10^{5}cells)	4	12.5	1	3	0	0	
-PCR assay:	**8**	**25**	**3**	**10**	**0**	**0**	
In Leukocytes	6	19	3	10	0	0	
In Plasma	2	6	0	0	0	0	

***EA**: ELISA arbitrary unit; **High Ab titer**: More than two fold (>160EA) of low titer (80EA) was taken as an indication of viral activity or reactivation among studied groups.
****Significant P value=<0.05**

Table 3 Presence of low and high CMV IgG Ab titer among certain age groups (<25Y, <50Y) in all subject groups

Age groups	<25 Y			>50 Y			P-Value
CMV titer Group	Total no.	<80 EA, >80-160	>160 EA*	Total no.	<80 EA, >80-160	>160 EA	
		No. (%)	No. (%)		No. (%)	No. (%)	
Leukemia	24	22 (91.7%)	2 (8.3%)	8	1 (12.5%)	7 (87.5%)	**<0.05****
Bladder Ca.	3	1 (33.3%)	2 (66.7%)	27	11 (40.7%)	16 (59.3%)	>0.05
Normal	18	1 (5.6%)	17 (94.4%)	8	2 (25%)	6 (75%)	>0.05

*EA: ELISA arbitrary unit. ****Significant P value=<0.05**

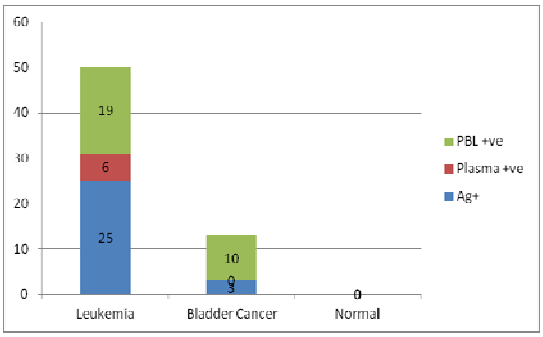

Fig. 2 Detection of HCMV by antigenemia and PCR assays in all subject groups

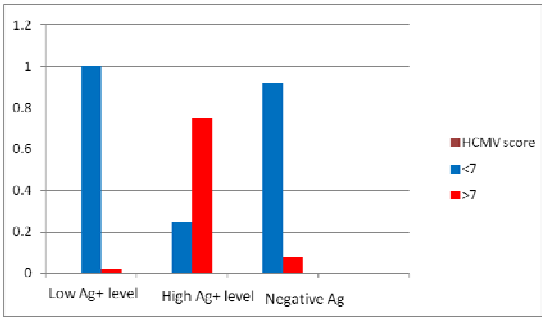

Fig 3 Correlation between severity of CMV disease and antigenemia level in leukemia patients

4. Discussion

Early and accurate diagnosis and reliable methods for monitoring CMV infection are essential not only for patients' managing [9], but also for epidemiological studies to understand host-virus relationship [10]. The conventional methods for the diagnosis of CMV infection/disease are viral isolation by viral culture, serology which includes CMV specific antigen and antibody detection, molecular method for detection of viral DNA from blood and clinical specimens. Viral isolation in tissue culture is the gold standard method but, it is labor intensive and takes time (24-48hrs) till the results are available. Hence, other rapid methods such as detection of pp65 antigen from peripheral blood leukocytes (antigenemia assay) and CMV DNA are preferred for diagnosis [4].

In accordance with previous Egyptian reports, the seropositive reactions to HCMV was 100% in Egyptian cancer patients, compared to >90 % among apparently healthy controls. Whereas, other study stated that HCMV seropositivity was 83% in immunocompromised patients with malignant and non malignant blood disorders [11]. Moreover, our results showed that the lowest percentage of high Ab titer (>160 EA) (28%) and the highest percentage of

low Ab titer (<80 EA) (15.6%) was among leukemia patients. This may reflect imbalance present in immune defense mechanism present in leukemia patients whose cell mediated immunity is impaired and favoring development of CMV infection and disease. These results were further confirmed by our observation which showed that only 37.5% (3/8) of leukemia patients positive for antigenemia had high HCMV Ab titer. Several previous studies supported our results which demonstrated that no serologic response in severely immunocompromised patients who suffered fatal disseminated CMV infection, since ability of patients to respond immunologically is obviously impaired [12, 13]. Also, in sensitive test used for detection of specific CMV-IgG, as concluded by Greijer et al., [14] who suggested the use of a combination of peptides from pp150, gB, and pp28 to give optimal and specific reactivity with CMV IgG.

Molecular methods considered to be the relevant diagnostic methods for detection of CMV DNA in various samples. In the present study, HCMV DNAemia (HCMV DNA in PBL) was detected in 19% (6/32) of leukemia patients, only 33%out of those patients had active HCMV infection and then they are at higher risk of developing CMV disease [15]. However, PCR test has the ability to detect minute amounts of nucleic acid in various clinical samples, and can detect the onset of CMV viremia 1 to 2 week prior to culture and antigenemia tests, its inherent sensitivity poses a problem because latent CMV genomes, which are present in leukocytes of practically all seropositive individuals, may be amplified [16]. In this context, Quantitation of CMV DNA in plasma and other biological samples is very useful for rapid diagnosis of infection and effective monitoring clinical course of disease and response to therapy.

The current study lack quantitation of HCMV DNA by real time PCR, but quantitation was performed by antigenemia assay. A valuable feature of the CMV antigenemia assay is that, it is rapid (2-4 hrs), quantitative, doesn't require special equipments, and antigenemia become positive 7 days before serologic signs of active infection. Therefore, antigenemia test is useful in early diagnosis, monitoring infection and antiviral treatment in immunocompromised patients [17]. Our results showed that 8 out of 32 leukemia patients (25%) were positive for HCMV by antigenemia assay. The quantitative nature of the antigenemia assay may give an estimate of viral load, and this may be useful for monitoring patients before, during, and after therapy.

When comparing results of our PBL-PCR and plasma PCR with antigenemia assay, sensitivity and specificity were found to be 75% and 100% for our PBL-PCR and 25% and 100% for our plasma PCR. False negative results obtained by PBL-PCR could be due to several reasons. First, sensitivity of primers used for amplifying CMV was low [18]. Second, Genetic variations and variability of clinical strains of HCMV may affect performance of PCR. Third, Presence of inhibitory factors in tested specimens. In addition, the six leukemia patients who were positive for antigenemia and were not viremic can be explained by early infection (new strain) or reactivation of HCMV has been detected. This was consistent with explanation of Boeckh et al. [19] who found that in the early stage of reactivation detection of DNA and pp65 likely represents mainly phagocytosis of virus DNA originating from replicating CMV-infected cells such as endothelial cells, which can be detected in peripheral blood in patients with progressive infection. That CMV DNA often cannot be detected in plasma during this phagocytosis process. This also was demonstrated by Lübeck et al [20] who observed that antigenemia preceded viremia by 3-9 days. Therefore, early positive or rising antigenemia levels may signal the onset of active CMV disease and allow early preemptive therapy to be initiated, particularly in transplant recipients [21].

Moreover, when comparing level of antigenemia with severity of HCMV disease, our results showed that 75% of leukemia patients with high antigenemia level had severe CMV disease as estimated by plotkin's scoring system. This was in consistent with previous report which showed that antigenemia rate which is one of the risk factors for development of fatal outcome of CMV disease in lymphoma patients (median number of CMV infected cells per 1000,000 WBCs was higher in patients with CMV-disease compared to those with antigenemia (median 18 versus 5 cells). Also, future research will need to focus on the implementation of real time PCR to achieve standardized, reproducible results on larger samples of cancer patients hoping to reach a cut off for diagnosing active HCMV infection besides the antigenemia assay using monoclonal antibodies.

Conclusion

Prevalence of HCMV IgG was 100% among immunocompromised cancer patients. Presence of high HCMV Ab titer was lower in leukemia patients than solid tumor patients, but presence of low HCMV Ab titer was higher in leukemia patients than solid tumor patients indicating impairment in cell mediated and humoral immune response as a result of disease. Quantitative Antigenemia assay showed better results than qualitative PCR assay for detection HCMV infection. Also, high level of antigenemia pp65 was correlated with severity of HCMV disease. Treatment decisions in the clinic should therefore be taken with

caution, considering trends in viral load and the sensitivity of the methods used in individual laboratories, clinical manifestations, and not just absolute values recorded in a single test. So, further studies using more sensitive and specific technique (real time PCR) is recommended.

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