RAPID DIAGNOSIS OF CYTOMEGALOVIRUS IN THAI PEDIATRIC AIDS PATIENTS

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Abstract. Blood samples were collected from 100 pediatric AIDS patients for the detection of CMV in pp65-bearing leukocytes (PBLs) by immunoperoxidase staining (IP) and PCR. IgM antibody assay was performed to determine the correlation of antigen and antibody. IP and PCR can be used as methods for the early detection of CMV (prior to the presence of IgM antibody). The sensitivity and specificity of IP were 73% and 97% respectively. IP is superior to PCR in several ways: it is very easy to perform, less time consuming, less expensive, and does not require expensive instruments.

INTRODUCTION

Cytomegalovirus (CMV) is found all over the world. CMV infection can be chronic and subclinical; symptomatic infections occur with significant prevalence and constitute a public health problem that warrants attention (Alford *et al*, 1990). Clinical CMV infection is usually found in newborn and immunocompromised hosts (Hodinkia, 1999). Significant morbidity and mortality among immunocompromised hosts *eg* organ transplantation recipients and those with the acquired immunodeficiency syndrome (AIDS), has been reported (Dworkin and Drew,1989; Hodinkia, 1999).

In patients with AIDS, invasive clinically significant CMV infections may be found in most organ systems, especially the CNS. Retinitis is the commonest ocular infection and is a sight-threatening condition in HIV-infected individuals (Britt and Alford, 1996; Britt, 2000). Patients with CMV retinitis are often asymptomatic and may go on to develop serious complications, including retinal detachment and blindness, if their disease is not recognized or treated promptly.

Although antiviral prophylaxis has led to a reduction in both the morbidity and mortality of CMV disease in recent years, the toxicity and the high cost of treatment remain as significant problems. Highly sensitive and qualitative methods of identifying patients at risk of CMV disease are needed.

Polymerase Chain Reaction (PCR) has been found to be the most sensitive method for the detection of CMV in blood, pp65 antigenemia is also considered to be a sensitive test, with a high predictive value when pp65-bearing leukocytes (PBLs) are present (Bowen *et al*, 1997; Dodt *et al*, 1997; Francisci *et al*, 1997; Mazzulli *et al*, 1999). CMV pp65 antigenemia levels are correlated with CMV disease in patients with AIDS (Mazzulli *et al*, 1993).

The aims of this study were to determine the presence of CMV in PBLs by both immunoperoxidase staining (IP) and PCR and to compare these two methods. The correlations of IgM antibody with IP and PCR was also to be determined. The intended benefit of this study was the establishment of a new

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method for routine diagnosis of CMV infection for use in small laboratories in provincial areas of Thailand that would enable physicians to make appropriate treatment decissions.

MATERIALS AND METHODS

Five milliliters of blood were collected from each of 100 pediatric AIDS patients of the Queen Sirikit National Institute of Child Health, Bangkok, between June 2000 and February 2001. The patients were aged between 1 month and 9 years. The blood samples were divided into two tubes: the first tube contained 2ml of blood with EDTA for the detection of CMV by IP and PCR; the second tube contained the remaining clotted blood, from which the serum was separated and used for the detection of IgM antibody by an ELISA method.

Immunoperoxidase staining

EDTA blood samples were mixed with isoprep, centrifuged, and washed twice with RPMI medium (GIBCO, USA). Approximately 10μl of PBLs were smeared onto glass slides, air dried, wrapped in aluminum foil, and kept at -70ºC for staining. Uninfected HEL cells and cells infected with CMV strain AD169 were used as negative and positive controls respectively.

The smears were fixed with formaldehyde, permeabilized with 1% Nonidet P-40 solution for 10 minutes and incubated with 3% H_2O_2-methanol for 5 minutes at room temperature. The monoclonal mouse anti-cytomegalovirus AD169 clone AAC10 (DAKO, Denmark) was added to the slides, which were then incubated for 30 minutes at room temperature, washed 3 times with PBS, and incubated with peroxidase conjugate and rabbit mouse immunoglobulins (DAKO, Denmark) for 30 minutes; the slides were then washed again with PBS. The substrate solution of 3-amino, 9-ethyl-carbazol was then added for 10 minutes followed by counterstaining with hematoxylin and mounting in glycerol-gelatin. The numbers and morphology of CMV-antigen-positive (CMV Ag^+) cells were determined by microscopy using a 25x objective; a sample with more than 5 CMV Ag^+ cells per slide was considered to be a positive case (van der Bij *et al*, 1988).

PCR and nested PCR

DNA was extracted from leukocyte nuclei by digesting 100 μl of each sample with 2 μl of proteinase K (Promega, USA) and 100 μl of digestion lysis buffer (DB). Amplification of DNA was performed with a total volume of 50 μl. The reaction mixture consisted of 10x reaction buffer, 15 mM $MgCl_2$, 200 μM of deoxynucleotide triphosphate (dNTP), 25 units of *Taq* (Thermus aquaticus) DNA polymerase, and 0.4 μM of primer (5′-GTCCTCTGCCAA GAGAAAGATGGAC-3′). The final volume was adjusted to 1 amplification cycle, consisting of sample denaturing at 94ºC for 1 minute, primer annealing at 65ºC for 2 minutes, and primer extension at 72ºC for 1 minute; the amplification was repeated during 34 further cycles. The products of the first amplification were transferred to a new 45 μl reaction mixture with a nested set of primers (5′-TCTCC TGTATGTGACCCATGTGCTT-3′); the amplification mixture was subjected to an additional 35 cycles, after which the amplification products were analyzed by 3% agarose gel (with ethidium bromide) electrophoresis for 30 minutes. The gel was photographed under UV illumination.

ELISA

IgM antibody assay was performed using the ELISA method described by Tantivanich (1999); the cut-off point titer was 1:160.

RESULTS

One hundred pediatric AIDS patients who were suspected of having contracted HIV infection from their mothers, were enrolled in this study to determine the presence of CMV by IP, PCR and IgM antibody: their baseline characteristics are given in Table 1. Twenty-

Table1
Baseline characteristics of study population.

Characteristics	Value
No. of pediatric AIDS patients	100
No. symptomatic	67
No. asymptomatic	33
Median age (months)	47
No. of males	45
No. of females	55
Median CD_4 lymphocyte count (No. of cells/mm^3)[a]	549
Median CD_8 lymphocyte count (No. of cells/mm^3)[b]	1,224

[a]Median CD_4 lymphocyte count was calculated from 81 pediatric AIDS patients.
[b]Median CD_8 lymphocyte count was calculated from 80 pediatric AIDS patients.

Table 2
Number (%) of positive results by IP, PCR, and IgM antibodies among symptomatic and asymptomatic patients.

	Symptomatic (%)	Asymptomatic (%)	Total (%)
IP	11 (11)	0	11 (11)
PCR	11 (11)	0	11 (11)
IgM	7 (7)	3 (3)	10 (10)
Total	29 (29)	3 (3)	32 (32)

Table 3
Age distribution of CMV infection detected by IP, PCR, and IgM antibody.

Age groups	No. (%) of positive results by		
	IP (%)	PCR (%)	IgM (%)
1-6 months	5 (5)	6 (6)	1 (1)
7-12 months	1 (1)	2 (2)	0
1-2 years	0	0	0
2-5 years	4 (4)	3 (3)	6 (6)
6-12 years	1 (1)	1 (1)	3 (3)
Total	11 (11)	12 (12)	10 (10)

Table 4
Sex distribution of CMV infection detected by IP, PCR and IgM antibody.

Sex	Number	No. (%) of positive results by		
		IP (%)	PCR (%)	IgM (%)
Male	45	6 (13.3)	6 (13.3)	2 (4.4)
Female	55	5 (9.1)	5 (9.1)	8 (14.6)

Table 5
Comparison of results by IP and PCR.

IP	PCR		Total
	Positive	Negative	
Positive	8	3	11
Negative	3	86	89
Total	11	89	100

two were positive by one or more methods and 78 were negative by all three methods. Two (2%) gave positive results by all three methods, 8 (8%) gave positive results by IP and PCR. IP and PCR gave positive results only in symptomatic patients, while IgM antibody could be detected in both symptomatic and asymptomatic patients. The numbers of symptomatic and asymptomatic AIDS patients with positive CMV by IP, PCR and IgM antibodies are shown in Table 2. The age and sex distributions of CMV infection detected by all three methods are shown in Tables 3 and 4: most of the positive results were found in the 1-6 months and 2-5 years age groups; there was no significant difference in the results according to sex. Comparison of the results abtained by IP and PCR is given in Table 5: eleven (11%) were found to be positive by both methods. Among these, 3/11 were found to be negative by PCR and 3/11 were found to be negative by IP. Correlations of IP, PCR and IgM antibody are shown in Table 6: there was good correlation between IP and PCR; there was no correlation between IP and IgM antibody and between PCR and IgM antibody.

Table 6
Correlation of IP, PCR and IgM antibody.

IP	PCR	IgM antibody	Number	%
+	+	+	2	2
+	+	-	6	6
+	-	+	0	0
+	-	-	3	3
-	+	-	3	3
-	+	+	0	0
-	-	+	8	8
-	-	-	78	78
Total			100	100

Age-specific $CD4^+$ T-lymphocytes in symptomatic and asymptomatic AIDS patients are shown in Table 7: there was no correlation between the number of $CD4^+$ T-lymphocytes and the positive CMV results by IP, PCR and IgM antibody; the $CD4^+$ T-lymphocytes count with positive IgM antibody ranged from 20 to 534; for IP and PCR the count was 8 to 2,898.

The positive results by IP and PCR were classified as indicating severe suppression as defined in the immunological categorization of the Center for Diseases Control (Thailand), which is based on the age-specific $CD4^+$ T-

Table 7
Age-specific CD_4^+ T-lymphocyte count (cell/mm^3) among 81 symptomatic and asymptomatic AIDS patients.

Age	CD_4^+	Symptomatic	Asymptomatic
< 12 months	≥1,500	6	1
	750-1,499	3	1
	<750	9	0
1-5 years	≥1,000	4	6
	500-999	1	7
	<500	13	8
6-12 years	>500	0	3
	200-499	0	2
	<200	12	5

Age specific CD_4^+ T-lymphocyte counts were classified by the CDC in 1997.

Table 8
Comparison of the diagnostic values of the pp65 antigenemia assay for CMV disease in AIDS patients.

Study population (AIDS patients)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	References
174	91	65	47	95	Bek *et al*, 1996
200	92	88	-	-	Dodt *et al*, 1997
49	85	64	46	92	Fox *et al*, 1998
62	81	72	-	-	Francisci *et al*, 1997
238	92	89	-	-	Mazzulli *et al*, 1999
36	100	86	-	-	Mazzulli *et al*, 1993
24	33	100	-	-	Pannuti *et al*, 1996
138	100	50	-	-	Walmsley *et al*, 1998
22	100	64	-	-	Wetherill *et al*, 1996
296	80	84	-	-	Wattanamano *et al*, 2000
100	73	97	73	97	This study

lymphocyte counts. The sensitivity and specificity of IP were 73% and 97% respectively.

DISCUSSION

CMV causes important clinical infection in immunocompromised HIV-seropositive patients: high morbidity and mortality are associated with retinitis, colitis and pneumonitis. The drugs that are available for treatment of CMV infection are at their most effective during the early stage of the disease; it follows, therefore, that new, rapid, and sensitive methods of early diagnosis are needed.

In this study, IP and PCR gave positive results only in symptomatic patients: these results may indicate that symptomatic AIDS patients have CMV co-infection and that both methods are adequate for use as standard methods for the diagnosis of CMV in pediatric AIDS patients. These two methods can also detect CMV prior to the presence of IgM antibody. These results were similar to the results reported by Amarapal *et al* (2001a;b), who used IP for the diagnosis of CMV in blood donors and neonate patients.

The positive results by all three methods indicated active infection because we could detect the antigen and the antibody simultancously; this type of infection can be either a primary or a recurrent infection. In pediatric AIDS patients, the presence of CMV in PBL determined by IP and PCR indicate early CMV infection, since the presence of CMV-infected leukocytes is the first indication of an active CMV infection; CMV antigenemia is a good predictor of CMV disease in AIDS patients (Torrus *et al*, 1999). CMV IgM antibodies accompanied by negative IP and PCR may be due to primary infection, with undetectable intraleukocyte CMV, or may have been false positives, due to a cross-reaction with herpes simplex virus type 6 and 7 (Osman *et al*, 1997). The positive results by IP and PCR in the 1-6 months age group indicated congenital or perinatal infection while in the 2-5 years age group they indicated primary or recent infection.

The prevalence of CMV by IgM antibody and PCR in this study was higher than in the previous studies by Tantivanich *et al*, (1999) and Temcharoen *et al* (1999); this may be due to differences in the size of the populations studied and the kinds of samples used. In this study, the samples were collected from pediatric AIDS patients, while Tantivanich *et al* (1999) and Temcharoen *et al* (1999) collected the samples from HIV-seropositive patients who did not have AIDS-defining illness.

The results of IP in this study are similar to those of previous studies, as summarized in Table 8.

The good correlation between the positive results of IP and PCR indicates that both IP and PCR can be used to detect the presence of CMV in leukocytes. The absence of IgM antibody in the presence of positive IP and PCR indicates that IP and PCR are the best methods for the detection of CMV infection, because these methods can detect the presence of the virus prior to the appearance of IgM antibody and do not have to rely on CD_4 count. IP should be introduced for use as a routine diagnostic method: the advantages of the IP technique are low cost (the estimated cost per test was about 2-3 times lower than that of PCR), ease of performance, and comparative rapidity; no expensive instruments or especially experienced staff are required.

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