# RAPID DIAGNOSIS OF CYTOMEGALOVIRUS INFECTION IN CONGENITAL NEONATES

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**Abstract.** Immunostaining was compared with PCR for diagnosis of congenital CMV infection. IgM and IgG antibody assays were also performed in parallel. Immunostaining gave sensitivity and specificity of 60% and 97% respectively. Correlations among immunostaining, PCR and the presence of IgM antibody was reported. Immunostaining can be used for early diagnosis of congenital CMV infection in parallel with detection of IgM antibody.

## INTRODUCTION

Cytomegalovirus (CMV) has been found all over the world. Most of the CMV infections are asymptomatic but the clinical problems usually found in congenital neonates and immunocompromised patients (Pass *et al,* 1980). The frequency of congenital CMV varies in different countries depending on antibody prevalence and socioeconomic status as well as on the ill-defined factor (Barbi *et al,* 1996). In Thailand, congenital CMV infection has been reported to be1.9% and most of the Thai population had antibodies against CMV (Tantivanich *et al,* 1980, 1999). Therefore, CMV infection, especially in congenital neonates, Should be considered as one of the important health problems among infectious diseases. The high mortality rate due to CMV infection can be prevented by early antiviral treatment and the effective treatment requires an early diagnosis (Williamson *et al,* 1982).

Diagnosis of congenital CMV infection relies on virus isolation from urine collected in the first 3 weeks of life. This method is time consuming and is not sufficient to diagnose acute infection. At the present time, PCR is the best diagnostic method but it requires experienced person to perform the test, and it is still too expensive to be used as a routine diagnosis in small laboratories. In this study, prevalence of CMV infection in congenital neonates has been assessed by comparing immunostaining method with PCR. IgM and IgG antibody analysis was preformed in parallel.

## MATERIALS AND METHODS

Two hundred and thirty-six urine samples, EDTA treated blood, and clotted blood were collected from the newborn infants with suspected CMV infection at Phramongkutklao Hospital and Queen Sirikit Institute of Child Health, Bangkok. For the control groups, the same kinds of specimens were collected from 140 newborn infants at Rajvithi Hospital. The urine samples were centrifuged and adjusted to pH 7.0 by $NaHCO_3$ then boiled for 10 minutes and cooled before being kept at -70ºC until used for detection of CMV by PCR.

For immunostaining, EDTA treated blood was mixed with isoprep, centrifuged, and washed twice with RPMI medium (GIBCO, USA).

Approximately 10 μl of cells were smeared on glass slides, air-dried, wrapped in aluminum foil and kept at -70ºC for further staining. Uninfected HELA cells and cells infected with CMV strain AD 169 were used as negative and positive controls, respectively.

Sera were separated from clotted blood and stored at -20ºC for detection of IgM and IgG antibodies.

**PCR and nested PCR**

Amplification of CMV DNA in urine samples were performed in a total volume of 50 μl. The reaction mixture consisted of 10x reaction buffer, 15mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTP), 25 units of *Taq* DNA polymerase, 0.4 μM of primer (5′-GTCCTCTGCCAAGAGAAAGATGGAC-3′). The final volume was adjusted to 50 μl with distilled water and overlaid with 20 μl of mineral oil. The reaction mixture was subjected 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 34 more cycles. The products of the first amplification were transferred to a new 45 μl reaction mixture with nested set of primers (5′-TCTCCTGTATGTGACCCATGT GCTT-3′). The amplification mixture was subjected to an additional 35 cycles. After that, the amplification products were analyzed by 3% agarose gel electrophoresis, containing ethidium bromide for 30 minutes. The gel was photographed under UV illumination.

**Immunostaining**

The smear slides were fixed with formaldehyde, permeabilized with 1% Nonidet P-40 solution for 10 minutes and incubated with 3% methanol-$H_2O_2$ for 5 minutes at room temperature. The monoclonal mouse anti-cytomegalovirus AD 169 clone AAC 10 (Dako, Denmark) was added to the slides, incubated for 30 minutes at room temperature, washed 3 times with PBS, and incubated with peroxidase conjugate, rabbit anti-mouse immunoglobulin (Dako, Denmark) for another 30 minutes. The slides were washed again with PBS, then the substrate solution of 3 amino, 9 ethylcarbazal was added for 10 minutes followed by counterstaining with haematoxylin and mouthing in glycerol-gelatin. The numbers and morphology of CMV-antigen positive cells were examined under a microscope with a 25x-magnification objective; more than 5 CMV-$Ag^+$ cells per slide was assumed to identify a positive case (van der Bij *et al*, 1988).

**Serological studies**

IgG and IgM antibodies were detected by using ELISA as described previously by Tantivanich *et al* (1999).

## RESULTS

Two hundred and thirty-six neonates with suspected CMV infection were examined for the presence of CMV by using PCR, immunostaining, and IgM and IgG antibodies. These neonates were found to have various clinical symptoms as demonstrated in Table 1. Twenty-three neonates (9.3%) had congenital CMV infection. Among those 60.9% neonates had jaundice and gave positive results by PCR

Table 1
Clinical symptoms of suspected 236 congenital CMV infection.

| Clinical symptoms | Numbers |
|---|---|
| IUGR | 15 |
| Hepatomegaly | 5 |
| Microcephaly | 7 |
| Jaundice | 190 |
| SGA | 1 |
| Skin rash | 2 |
| Oligohydramnios | 3 |
| Polyhydramnios | 3 |
| Pneumonia | 3 |
| Hydrocephalus | 2 |
| Thrombocytopenia | 1 |
| Cloudy cornea | 1 |
| Hydrops fetalis | 2 |
| Malformations of ears and eyes | 1 |

and immunostaining while of those who had IUGR, polyhydramnios and hepatomegaly 8.7% gave positive results (Table 2). The correlation among PCR, immunostaining, and the presence of IgM antibodies was illustrated in Table 3. Among these who gave positive results by PCR or immunostaining, 4/23 (17.4%) gave positive results by PCR and IgM antibody; 2/23 (8.7%) had no IgM antibody presence. Eleven patients (11/23 or 47.8%) had only positive results by PCR, of which 2 of them had IgG antibody. The remaining neonates (213/236, 90.2%) gave positive results by both PCR and immunostaining; of these 42/213 (19.7%) had IgG antibody with the titer ranging from 1:80-1:320.

All of the normal neonates from healthy mothers gave negative results by PCR, immunostaining and IgM antibody, but IgG antibodies were detected in 29 neonates. Among these, 26 had antibody titers ranging from 1:80 to 1:160 and 3 had the titers of 1:320 to 1:640.

Sensitivity and specificity of the immunostaining method when using PCR as a gold standard were 60% and 97% respectively.

## DISCUSSION

CMV is commonly found as congenital infection. However, only very small numbers of infected infants have symptoms at birth with various clinical syndromes. Diagnosis of congenital CMV can be achieved by detection for the presence of virus by tissue culture, by DNA analysis, or by detection of specific IgM antibody. In this study, PCR, immunostaining and IgM antibody were determined and compared. It was found that PCR is the most sensitive method. The results in this study showed good correlation between PCR, immunostaining and the presence of IgM antibody. The absence of IgM antibody with positive PCR and immunostaining may indicate that both PCR and immunostaining are the best methods for detection of CMV infection. These methods can detect the presence of the virus

Table 2
Clinical symptoms with CMV positive by PCR and immunostaining.

| Clinical symptoms | Number | Percents |
|---|---|---|
| IUGR low birth weight (1,200g) | 2 | 8.7 |
| IUGR normal birth weight | 2 | 8.7 |
| Jaundice | 14 | 60.9 |
| Microcephaly | 1 | 4.3 |
| Polyhydramnios | 2 | 8.7 |
| Hepatosplenomegaly | 2 | 8.7 |
| Total | 23 | 100.0 |

Table 3
Correlation of PCR, immunostaining IgM and IgG antibodies of congenital CMV infection.

| PCR | Immunostaining | IgG titer | IgM titer | Numbers |
|---|---|---|---|---|
| + | + | 1:160 | 1:80 - 1:160 | 4 |
| + | + | - | - | 2 |
| + | - | 1:80 | - | 2 |
| + | - | - | - | 9 |
| - | + | - | - | 6 |
| - | - | 1:80 - 1:320 | - | 42 |
| - | - | - | - | 171 |

prior to the presence of the IgM antibody. The presence of IgG antibody with negative results by PCR and immunostaining indicated the passive transfer antibodies from their mothers with no CMV infection.

Even though immunostaining is less sensitive than PCR, the specificity is high enough to recommend it for diagnosis of congenital CMV. The reasons for low sensitivity may due to (1) keeping the EDTA blood more than six hours which can diminish CMV antigenemia levels; (2) low volume of EDTA blood can lead to a low number of CMV infected cells. If these problems can be solved, the immunostaining method should replace other methods that are currently used for rapid diagnosis of CMV infection or are performed in parallel with detection of IgM antibody if the physician wants to know whether it is a recent or a past infection. This method is less time consuming, very easy to perform, requires no experienced workers and is cheap. In addition, this method is sensitive and can detect the presence of the virus prior to the presence of the IgM antibody.

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