Urine polymerase chain reaction as a screening tool for the detection of congenital cytomegalovirus infection

Y Schlesinger, D Halle, A I Eidelman, D Reich, D Dayan, B Rudensky, D Raveh, D Branski, M Kaplan, V Shefer, D Miron

Objectives: To define the incidence of congenital cytomegalovirus (CMV) infection in a defined population in Israel as diagnosed by urine polymerase chain reaction (PCR), and to assess the utility of this method for screening for congenital CMV infection.

Design: A convenient sample of urine specimens from asymptomatic newborns were subjected to CMV PCR. Positive results were validated by urine tube culture and by determination of serum CMV IgM antibodies. Maternal CMV IgG was determined in a representative sample of mothers. Newborns with positive urine specimens underwent full clinical evaluation. Epidemiological characteristics of the mothers were extracted from the medical records.

Settings: Two medical centres in Israel with different population characteristics.

Patients: A total of 2000 newborns (1000 in each medical centre).

Main outcome measure: Presence of CMV DNA in the urine.

Results: Despite significant epidemiological differences between the populations in the two hospitals, the CMV seroprevalence was similar, 80.5% and 85%. Fourteen of the 2000 newborns screened (0.7%) were PCR positive. Urine culture was positive in nine of 10 specimens; IgM was positive in only two of 13 newborns with positive PCR. Eleven newborns underwent full or partial evaluation, and only one (9%) was symptomatic.

Conclusions: The incidence of congenital CMV infection in the study population was 0.7%; over 90% were asymptomatic. Urinary CMV PCR is a reliable, rapid, and convenient method, and thus may serve as a screening tool for the detection of congenital CMV infection.

Congenital cytomegalovirus (CMV) infection is currently the leading cause of congenital infection world wide, occurring in 0.2–2.2% of live births. Over 90% of neonates with congenital CMV infection appear normal at birth. However, 10–20% of such infants will suffer from late central nervous system sequelae, primarily sensory-neuronal deafness. Early detection of hearing deficits and intervention is mandatory to minimise subsequent neurodevelopmental dysfunctions. In addition, the preliminary results of the randomised, placebo controlled study of the National Institute of Allergy and Infectious Diseases (NIAID) (Anti Viral Study Group) of ganciclovir treatment of symptomatic newborns with congenital CMV infection suggest that ganciclovir may be superior to placebo in preventing hearing deterioration in these patients. As this study did not examine asymptomatic newborns with congenital CMV infection, no simple extrapolation can be made from this study to asymptomatic patients. However, given the potential benefit of ganciclovir, its potential role in the treatment of this patient population should be explored. Hence, a simple method that would allow screening of asymptomatic infants with congenital CMV infection would be of interest.

Classically, the diagnosis of congenital CMV infection has been made by detection of the virus in the urine within the first three weeks of life. Currently, the best approach is to cultivate the virus from fresh urine in tissue culture. However, this technique is expensive and laborious and therefore not very suitable for mass screening. Polymerase chain reaction (PCR) has recently been shown to be a sensitive method for detecting CMV DNA in the urine and thus can be used to diagnose congenital CMV infection. As it can be performed on stored frozen urine specimens, it is a potentially suitable method for rapid screening of a large number of neonates. In contrast, serological diagnosis of congenital CMV infection (CMV IgM) has been shown in several studies to be an insensitive method.

The seroprevalence of Israeli women of childbearing age in a recent study of 6126 parturient women was 84.3%, leaving 15% of the women susceptible to primary CMV infection during pregnancy. Hence, routine screening for congenital CMV in Israel was deemed not to be justified. Given the pool of potentially CMV infected infants and the availability of the new PCR methodology, we initiated this study. The two primary aims were to (a) evaluate the diagnostic accuracy of the PCR technique and (b) delineate the incidence of congenital CMV infection in a defined population so as to evaluate the value of the PCR method as a mass screening tool.

PATIENTS AND METHODS

Study population

The study was conducted between 1 May 1998 and 31 August 31 1999 in two hospitals: Shaare Zedek Medical Center in Jerusalem (centre 1) and Ha’Emek Medical Center in Northern Israel (centre 2). These two medical centres were chosen because their populations differ in many epidemiological aspects from each other, and represent the heterogeneous population typical of the State of Israel. Two thousand neonates aged up to 3 days (1000 newborns each) were included. The ethics committees of the two participating hospitals approved the study. Informed written consent was obtained from the parents of each newborn.

Abbreviations: CMV, cytomegalovirus; PCR, polymerase chain reaction; BERA, brain stem evoked response audiometry
Basic data on participating mothers, obtained from the hospital records, included maternal age, ethnic group (Jewish or non-Jewish), and residency (urban or rural).

**Specimen collection and processing**
The selection of newborns from whom specimens were collected was based on convenience. Urine specimens were collected into a sterile urine bag attached to the perineum after thorough cleaning. All the urine specimens were processed in the Infectious Diseases Molecular Diagnostic Laboratory at Shaare Zedek Medical Center. The specimens from centre 2 were stored at 4°C, and transported on ice within one week to the laboratory. On arrival, they were labelled and boiled for 10 minutes at 100°C to denature proteins that may inhibit the PCR. All the samples were stored at 4°C, centrifuged, and the supernatants used for the PCR.

**PCR assay**
Common methods to prevent contamination and false positive results were used, including separate rooms for setting up the reaction and detection of the products, UV irradiation of all the reaction components except the primers, the Taq polymerase, and the urine specimens, and use of pluged tips. A negative control tube was added to each PCR run. One of two sets of primers was used for the screening. A positive result with either set was confirmed with the other.

The first primer set was:

5’-CCGCAACCTGTGGCCCATGG-3’
5’-CGTGTGCGTCGGCCGGG-3’.
This amplifies 139 bp from the gp64 late antigen region.

The second primer set was:

5’-AGCTGCTAGTGTAGGCAAGG-3’
5’-GAAAACTAGTCTCAGTGTAA-3’. This amplifies 147 bp from the 4th exon of the human CMV immediate early antigen.

The reaction mixture contained 12 pmol each primer, 200 μmol each dNTP, 0.2 units Taq polymerase (SR Products, Sevenoaks, Kent, UK), and buffer containing MgCl₂ at a final concentration of 1.5 mmol/l, supplied by the same company. A 1.2 μl sample of the urine to be tested was added to the reaction mixture to a final volume of 12.5 μl. The amplification was performed in a RoboCycler (Stratagene, La Jolla, California, USA). After initial heating at 95°C for two minutes, 32 cycles of 95°C for 30 seconds, followed by 65°C for 60 seconds were performed. The reaction was finished with 10 minutes at 65°C. The reaction products were run on a 3% agarose gel, and the urine viral load in congenital CMV is very high, and was recently determined to be 1.6 × 10⁹ copies/ml. Therefore the assay was deemed suitable for screening purposes.

**Serology**
Measurement of cord blood IgG antibodies against CMV was performed on 333 (33.3%) consecutive mothers in centre 1 and 283 (28.3%) consecutive mothers in centre 2, reflecting maternal CMV seroprevalence.

The serum of newborns with either positive urine PCR or tube culture for CMV in their urine specimen was tested for the presence of CMV IgM by antibody capture ELISA (DiaSorin, Saluggia, Italy).

**Clinical assessment**
Newborns with positive urine PCR infection were clinically assessed as follows:

• repeated complete physical examinations
• blood tests (complete blood count, kidney function tests, liver enzymes, CMV IgM)
• head ultrasonography or computed tomography scan for identification of brain calcifications
• retinal examination for diagnosis of chorioretinitis
• hearing evaluation by brain stem evoked response audiometry (BERA)

**Statistical analysis**
Data were analysed using the EpiInfo 6.0 software package. p Values were calculated by χ², and by the Fisher exact test where indicated because of small numbers. p < 0.05 was considered to be significant.

**RESULTS**
Overall, 2000 newborns were included in the study, of which 140 (7.2%) were premature (< 37 weeks gestation). There were differences in epidemiological aspects between the two centres with regard to ethnicity (Jews v Arabs) and residency (urban v rural). However, there was no significant difference in CMV seroprevalence between the two centres (80.5% and 85% in centres 1 and 2 respectively). Also, there was no difference in CMV seroprevalence between Jews and non-Jews, or between urban and rural residents (data not shown). As the rates were very similar, we combined the two groups and analysed them as one. Fourteen of the 2000 newborns (0.7%) were found to have CMV DNA in their urine, consistent with congenital CMV infection. Maternal epidemiological data were available for 1939 newborns. Table 1 shows the major characteristics.

Table 2 summarises the clinical characteristics of the newborns with congenital CMV infection. Urine of 10 of the 14 newborns with a positive urinary CMV PCR was cultured for CMV. The families of the other four either refused (two), were not available (one), or had left the country before the test was performed.
performed (one). Nine of the 10 cultures were positive for CMV. In the one culture negative case, three urine specimens collected on different occasions were strongly PCR positive with both primer sets, and culture, which was performed only once, was considered to be falsely negative. CMV IgM was measured in 13 infected infants and was found in only two neonates (15%), one symptomatic and one asymptomatic. Eleven of the 14 newborns with congenital CMV infection underwent a full or partial evaluation according to the study plan. Two families refused, and one patient could not be evaluated because his parents had left the country before results of the PCR testing became available. Only one infected newborn (7%) was symptomatic (No 10). He was born at term after an uneventful pregnancy with no prenatal evaluation. He was small for gestational age with a birth weight of 2510 g (3rd centile) and a head circumference of 28.5 cm (< 3%). He had hepatosplenomegaly and thrombocytopenia (80 000/\( \mu \)l) and a platelet count of 24 000/\( \mu \)l (3rd centile). His parents had a similar number of siblings with congenital CMV infection, which is much higher than that of hypothyroidism or phenylketonuria. He had transient mild hepatitis. The spectrum of the mass screening programme in many US states has been widened, and many states routinely screen asymptomatic newborns for various diseases, including congenital infections such as HIV and toxoplasmosis. Both the incidence of congenital CMV, which is much higher than that of hypothyroidism or phenylketonuria, and our results suggesting that urine PCR screening is convenient and reliable, makes CMV an attractive object of screening. However, the justification for routine screening may be questioned because of the lack of a proven mode of intervention. On the other hand, it may be argued that the knowledge per se of the risk of sensory-neuronal damage in the CMV infected newborn should prompt close monitoring and early diagnosis and intervention. Furthermore, the results of the randomised, placebo controlled study of the NIAID study noted superiority of ganciclovir over placebo in the treatment of symptomatic patients in preventing hearing deterioration. These results are premature, and as yet no firm recommendations have been made. Therefore the role of such treatment for asymptomatic children needs to be considered carefully.

**DISCUSSION**

The utility of PCR in place of urine culture and CMV IgM as a screening tool for congenital CMV infection is the most important finding of this study. Urine PCR was found to be very specific. We acknowledge that the sensitivity of the PCR assay was not directly assessed in this study, as urine cultures were not performed routinely on all the specimens. Furthermore, some investigators have questioned the validity of PCR results on urine specimens, because of the possible presence of PCR inhibitors in the urine. However, on the basis of our experiments showing 99% PCR positivity in 100 urine specimens spiked with as low as 20–30 viral copies of CMV, we consider that we have shown the assay methodology to have almost 100% sensitivity, and therefore confirmed its value and utility as a screening tool. Urine PCR has also been successfully used as a screening method by Demler et al and Tsai et al. In the future, screening may become even simpler, with the option of DNA extraction from Guthrie filter paper and automated amplification of CMV sequences. The CMV IgM antibodies were negative in all but one of the asymptomatic CMV infected newborns. Melish and Hanshaw have also found that only 50% of culture proven CMV infected newborns were IgM positive, and noted that the IgM results tended to be positive along with higher titres in the symptomatic newborns. These results are very important for clinicians, because, although IgM is known not to be fully sensitive, this method is still considered reasonable for the diagnosis of congenital CMV infection. We found a combined seroprevalence of 82.6%. This is similar to that of Stein et al, who found a seroprevalence of 84.3% in 6126 women of childbearing age in Israel. It seems that, despite major differences among different subgroups in this country, the overall CMV seroprevalence is similarly very high, with a consequent incidence of CMV infection. Assuming a 4% risk of primary CMV infection during pregnancy, 40% risk of fetal infection, and a 10% risk of early symptomatic infection, the 135 000 annual deliveries in Israel translate to 30–35 cases of symptomatic congenital CMV infection. A similar number of infants who appear normal at birth are expected to develop late neurological dysfunction, primarily hearing deficits. We found congenital CMV infection in 14 of 2000 newborns, and if the same rate applies to the remainder of the country, about 945 cases can be expected to occur annually, most of which probably being the result of re-activation of previous infection. With the new data of Bopp et al showing symptomatic infection in infants of mothers with re-activation rather than primary CMV infection during pregnancy, the number of symptomatic patients for whom routine screening is justified may be higher than that estimated by Stein et al.

The spectrum of the mass screening programme in many US states has been widened, and many states routinely screen asymptomatic newborns for various diseases, including congenital infections such as HIV and toxoplasmosis. Both the incidence of congenital CMV, which is much higher than that of hypothyroidism or phenylketonuria, and our results suggesting that urine PCR screening is convenient and reliable, makes CMV an attractive object of screening. However, the justification for routine screening may be questioned because of the lack of a proven mode of intervention. On the other hand, it may be argued that the knowledge per se of the risk of sensory-neuronal damage in the CMV infected newborn should prompt close monitoring and early diagnosis and intervention. Furthermore, the results of the randomised, placebo controlled study of the NIAID study noted superiority of ganciclovir over placebo in the treatment of symptomatic patients in preventing hearing deterioration. These results are premature, and as yet no firm recommendations have been made. Therefore the role of such treatment for asymptomatic children needs to be considered carefully.

**Table 2** Characteristics of infants with congenital cytomegalovirus (CMV) infection

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Sex</th>
<th>Ethnic group</th>
<th>Gest. age (weeks)</th>
<th>SGA</th>
<th>Microcephaly</th>
<th>CMV IgM</th>
<th>Urine culture</th>
<th>Head CT/US</th>
<th>Retinal exam</th>
<th>BERA</th>
<th>Blood tests†</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td>J</td>
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<tr>
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<tr>
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<tr>
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<td>Hepatitis</td>
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<tr>
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<td>ND</td>
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</tr>
<tr>
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<td>M</td>
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<td>N</td>
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<td>Positive</td>
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<td>Normal</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
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<td>28</td>
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<td>N</td>
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<td>Positive</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td>Normal</td>
</tr>
</tbody>
</table>

* Patients 1 through 10 are from centre 1; patients 11 through 14 are from centre 2.† Complete blood count, liver enzymes, renal function.

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Gest. age (weeks)</th>
<th>SGA</th>
<th>Microcephaly</th>
<th>CMV IgM</th>
<th>Urine culture</th>
<th>Head CT/US</th>
<th>Retinal exam</th>
<th>BERA</th>
<th>Blood tests†</th>
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</thead>
<tbody>
<tr>
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<td>Negative</td>
<td>Positive</td>
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<td>Normal</td>
<td>Normal</td>
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</tr>
<tr>
<td>NJ, non-Jewish</td>
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<td>N</td>
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<td>ND</td>
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<td>Normal</td>
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<td>Hepatitis</td>
</tr>
<tr>
<td>Gest, gestational</td>
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<td>Negative</td>
<td>Positive</td>
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<td>ND</td>
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<td>ND</td>
</tr>
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<td>Normal</td>
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<td>ND</td>
</tr>
<tr>
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<td>Negative</td>
<td>Positive</td>
<td>ND</td>
<td>ND</td>
<td>Normal</td>
<td>ND</td>
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<tr>
<td>US, ultrasonography</td>
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<td>N</td>
<td>Negative</td>
<td>Positive</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>exam, examination</td>
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<td>N</td>
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<td>Negative</td>
<td>Positive</td>
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<td>ND</td>
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<td>ND</td>
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<tr>
<td>BERA, brain stem evoked response audiometry</td>
<td>40</td>
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<td>N</td>
<td>Negative</td>
<td>Positive</td>
<td>ND</td>
<td>ND</td>
<td>Normal</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Note:**

9-fetal infection, and a 10% risk of early symptomatic infection, the 135 000 annual deliveries in Israel translate to 30–35 cases of symptomatic congenital CMV infection. A similar number of infants who appear normal at birth are expected to develop late neurological dysfunction, primarily hearing deficits. We found congenital CMV infection in 14 of 2000 newborns, and if the same rate applies to the remainder of the country, about 945 cases can be expected to occur annually, most of which probably being the result of re-activation of previous infection. With the new data of Bopp et al showing symptomatic infection in infants of mothers with re-activation rather than primary CMV infection during pregnancy, the number of symptomatic patients for whom routine screening is justified may be higher than that estimated by Stein et al.

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be fully studied. Hence, a screening methodology that can identify such potential asymptomatic patients should be welcomed.

In summary, this study illustrates the potential role of urine PCR as a screening method for the diagnosis of congenital CMV infection, and confirms the lack of sensitivity of the newborn IgM status for the same purpose. By using the urine PCR screening method, we were able to evaluate the magnitude of the problem in a defined population in Israel. These results may be the basis for future larger scale studies to evaluate the feasibility and effectiveness of a routine screening programme of newborns for the presence of congenital CMV infection.

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