Original Article

The prevalence of congenital and perinatal cytomegalovirus infections among newborns of seropositive mothers

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Abstract

Introduction: This study aimed to determine the prevalence of congenital and perinatal human cytomegalovirus (HCMV) infections among newborns in two major neonatal intensive care units (NICU) in Bahrain.

Methodology: One hundred newborns comprised of 84 preterm and 16 term babies admitted to the NICUs were enrolled in the study. During the first six weeks of life, urine and saliva was obtained from the babies weekly and serial breast milk samples were obtained from the mothers. Maternal serum HCMV IgG was measured. Virus isolation and detection was done by shell vial culture and nested PCR.

Results: Maternal HCMV IgG-seropositivity was 100%. Eight HCMV infections were detected comprising of three congenital and five perinatal infections. Congenital HCMV infection was found in preterm (2/84; 1.9%) and term (1/16; 6.3%) babies. HCMV DNA was detected in breast milk samples obtained during the first 10 days postpartum from all mothers whose babies had congenital HCMV. Forty-nine women provided breast milk samples between four and six weeks post-partum and HCMV DNA was detected in the breast milk of 11 women. Five (45.5%) of these eleven were mothers of babies with perinatal HCMV infection. There was no significant difference in the detection of HCMV using shell vial culture versus nested PCR method.

Conclusion: The findings indicate occurrence of congenital and perinatal HCMV transmission in this setting of high maternal seropositivity. The use of shell vial culture and PCR amplification for HCMV screening in the NICU for rapid detection of infection during the early postnatal period is recommended.

Key words: Human cytomegalovirus, neonatal, congenital infection, perinatal infection

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Introduction

Human cytomegalovirus (HCMV) represents one of the most common causes of congenital virus infection. Congenital HCMV infection is usually associated with the occurrence of primary maternal infection during pregnancy or reactivation or re-infection with a different HCMV strain [1]. Infants with congenital HCMV as a result of primary maternal infection have more severe sequelae and worse prognosis compared to babies whose mothers had reactivated or secondary infections. Preterm infants tend to show higher susceptibility to postnatal and perinatal infections [2]. However, most maternal infections with HCMV are not diagnosed because the virus usually produces few, if any, symptoms and tends to be reactivated intermittently without symptoms. In many countries, routine screening of women to determine their HCMV immune status prior to conception is not performed, partly because of unavailability of rapid and accurate diagnostic methodology and lack of guidelines for therapy [3]. Although a previous study has shown a high rate of seropositivity among pregnant women in Bahrain, the incidence of congenital and perinatal infection has never been determined [4]. This study was conducted to determine the prevalence of congenital and perinatal HCMV infections in neonatal intensive care units (NICUs) as well as assess the occurrence of viral shedding in breast milk of lactating mothers in a setting of high maternal seropositivity. In addition, we have compared a rapid culture method with genome amplification techniques for HCMV detection.

Methodology

Study design and population

The study was conducted in the NICUs of two major public hospitals in Bahrain, namely Salmaniya Medical Complex (SMC) and Jidhaffs Maternity Hospital (JMH), between June 2006 and February 2007. The study population was comprised of newborns (term and preterm) and their mothers. The inclusion criteria was preterm babies (< 34weeks gestational age at birth); term babies (> 34weeks gestational age at birth) with clinical features suggestive of congenital HCMV. The exclusion criteria included infants of HCMV seronegative mothers and preterm babies with seizures primarily diagnosed as being a consequence of severe birth asphyxia.

Congenital and perinatal infections were defined as described by Distefano *et al*. [5]. Detection of HCMV in urine and/or saliva in newborns by shell-vial culture and/ or polymerase chain reaction test during the first three weeks of life was considered congenital infection. Perinatal HCMV was diagnosed if HCMV was detected in urine and/or saliva by shell-vial culture and/ or polymerase chain reaction (PCR) test after the first three weeks of life.

The study was approved by the Research and Ethics Committees of the College of Medicine and Medical Sciences, Arabian Gulf University, Bahrain. Informed Consent was obtained from the parents or legal guardians.

Sampling procedure

For preterm babies, three urine and saliva samples were collected: the first one during the first 10days of life, the second at four weeks and the third at six weeks postpartum. For term babies, only one set of urine and saliva samples was collected and this was collected during the first 10 days of life.

Serial breast milk samples for HCMV screening were obtained from the mothers during the first 10 days postpartum and at four and six weeks postpartum. Peripheral blood was obtained during the first postpartum week for maternal HCMV screening. Additional clinical data was obtained from case files and laboratory records.

Sample collection, transportation and storage

Saliva samples were collected by swabbing the inside of the buccal cavity of newborns with a cotton-tipped applicator and transported in viral transport mediums (Diagnostic Hybrids, Athens, OH, USA).

Urine specimens were collected without preservatives and transported on melting ice to the laboratory where they were divided into two aliquots. One aliquot was frozen at -80ºC for individual and pooled PCR testing and 2 ml of the other aliquot was transferred to viral transport medium and processed for individual shell vial culture.

Expressed breast milk (2-5 ml) was collected in sterile containers and transported on melting ice to the laboratory for immediate processing. Specimens were centrifuged at 1500 rpm for 15 minutes and the creamy fat globules layer discarded. The milk wheys were filtered using a 0.2µm sterile filter and stored at -80 ºC.

Sample processing

All urine, saliva and breast milk samples were tested for HCMV DNA by nested PCR; only urine and saliva samples were tested using the shell vial culture technique.

Shell vial culture was performed as follows: commercially prepared shell vials containing frozen monolayers of Hs27 (human foreskin fibroblasts) on 12-mm round cover slips in freeze mediums were obtained from Diagnostic Hybrids, Athens, Ohio, USA. Thawing, rinsing and inoculation of re-feeding media was performed using manufacturer provided reagents and instructions. Duplicate shell vials were inoculated with 0.5 ml of clinical specimens, centrifuged at 700 g for 45 minutes at 25ºC and 1 ml of pre-warmed culture re-feeding medium added. Vials were incubated in 5% CO2 at 37°C. For each specimen, one vial was stained after 16 to 24 hours and the second vial after 40-48 hours. Simultaneous cultures with known HCMV strain and uninoculated vials were used as positive and negative controls respectively. Cell quality was assessed by microscopic examination of one shell vial in each new batch.

The shell vial fixation and staining procedure was performed as follows: Cultured shell vials were rinsed 2-3 times with 1 ml of phosphate buffered saline (PBS) and fixed with 1 ml of cold acetone for 10 minutes at room temperature. Staining was carried out using the Cytomegalovirus Immediate Early Antigen Indirect Fluorescent Antibody Test kit according to the manufacturer instructions (Diagnostic Hybrids, Athens, OH, USA). Stained cover slips were examined immediately. Positive and negative control slides provided by the manufacturer were stained concurrently as controls. Samples were considered HCMV positive if at least one intact cell

with typical intranuclear immunofluorescence staining was present.

Nested PCR was performed as follows Specimens were thawed and pooled for DNA extraction and nested PCR. The pooling was done according to the results obtained from the shell vial cultures. Each type of specimen (urine and saliva) which was HCMV negative by shell vial culture was pooled. Sets of five samples were pooled (40 µl from each) for DNA extraction and nested PCR. When nested PCR results were positive for a pooled lot, then a fresh aliquot of each sample in that pool was individually thawed and processed for DNA extraction and nested PCR. HCMV DNA extraction was conducted using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmBH, Mannheim, Germany) according to the manufacturer's instructions.

Nested PCR for HCMV DNA was conducted as previously described using primers targeting the glycoprotein B (gB) gene [5,6]. The outer primers sequence targeting the 150-bp length region of the HCMV gB (gB1)5'-GAGGACAACGAAATCCTGTTGGGCA-3' and (gB2) 5'-GTCGACGGTGGAGATACTGCTGAGG-3' as well as the inner primers targeting the 100-bp length region of the HCMV gB sequence (gB3) 5'-ACCACCGCACTGAGGAATGTCAG-3' and (gB4) 5'-TCAATCATGCGTTTGAAGAGGTA-3' were obtained from Thermo Electron GmbH, Karlsruhe, Germany. Each amplification run contained a positive control of purified viral DNA extracted from and sterile RNase free distilled H_2O as a negative control. A sample was considered positive for HCMV when it yielded the characteristic band corresponding to 100 bp (first amplification) and 150 bp (second amplification).

Statistical analysis

Data were entered and analyzed using SigmaStat version 3.5 (Systat Software, Inc, San Jose, CA, USA).

Results

One hundred newborns (84 preterm and 16 term newborns) and 83 mothers (67 mothers of singletons, 15 mothers of twins, and one mother of triplets), were enrolled in the study. The principal indication for NICU admission was prematurity, intrauterine growth restriction, respiratory distress, sepsis, and anaemia. All the mothers were HCMV IgG positive; none had HCMV IgM. The mean age of the mothers in the study was 24 years (range: 15 to 45 years). Figure 1 shows the workflow chart of sample collection and processing.

The effectiveness of PCR and shell vial culture for detection of HCMV in urine and saliva was compared. During the study period, a total of 153 saliva and 150 urine specimens were examined by PCR and shell vial culture (Figure 1). Of these, 16 (8 urine and 8 saliva) specimens were positive by DNA amplification and 14 (8 urine and 6 saliva) specimens were positive by viral culture. As such, the rate of isolation of HCMV by either of the techniques was similar when using urine samples. However, for saliva, the molecular method correctly identified two positive specimens which were missed by shell vial culture. There was 100% agreement between the PCR results of urine and saliva specimens. For shell vial culture, comparison of positive urine and saliva specimens showed 97.3% agreement. Figure 2 shows the shell culture positive slides.

Of the 100 babies enrolled, eight were found to have HCMV infection. Seven of these babies were born to mothers under the age of 30 years. Following the defined criteria, 3% (n/N = 3/100) of newborns were categorized as congenital HCMV. This comprised of preterm: (n/N = 2/84; 1.9%) and term (n/N = 1/16; 6.3%) babies. Both urine and saliva specimens confirming congenital HCMV infection were obtained before transfusion. Five babies (all preterm) had perinatal infections.

Seventy-eight HCMV seropositive mothers, who breastfed their infants, provided breast milk for testing during the first 10 days postpartum. From this cohort, 49 women also provided samples at 4 and 6 weeks (Figure 1). HCMV DNA was detected in 4% (n/N = 3/78) of samples collected within 10 days postpartum. All three mothers with HCMV DNA shedding in breast milk within 10 days postpartum had babies with congenital HCMV infection. Among the 49 women who provided breast milk at 4-6 weeks postpartum, the breast milk from 11 women (22%) was positive for HCMV DNA. Five of these women were the mothers of the five preterm babies who developed perinatal HCMV infection (n/N = 5/11; 45.5%).

Jaundice, sepsis, and respiratory distress syndrome are the most common clinical findings found in 62.5%, 50% and 37.5% respectively of the HCMV positive babies. Thrombocytopenia and atypical lymphocytosis were present in 37.5% and

Figure 1. Flow chart for specimen collection and processing for HCMV detection

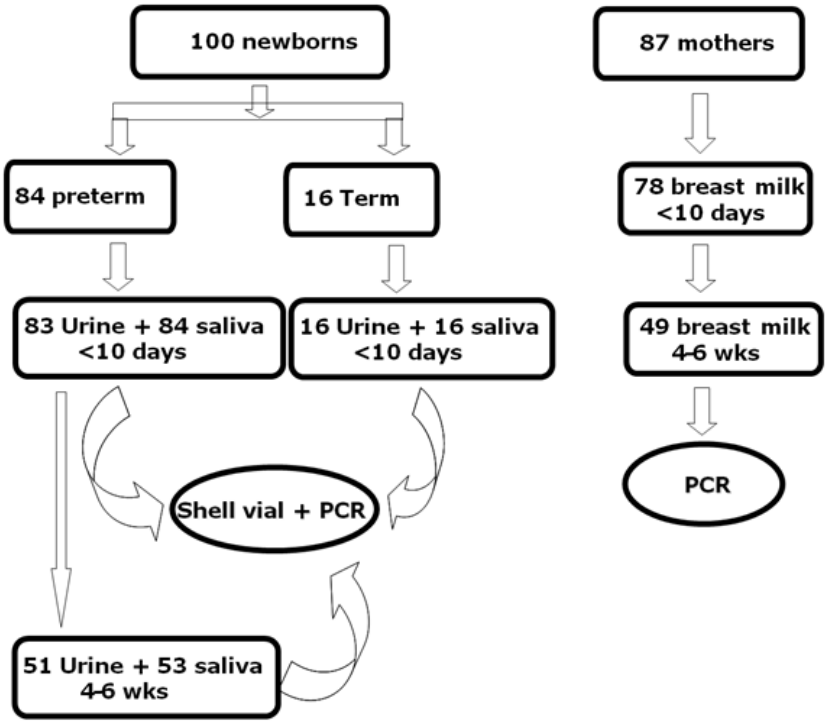
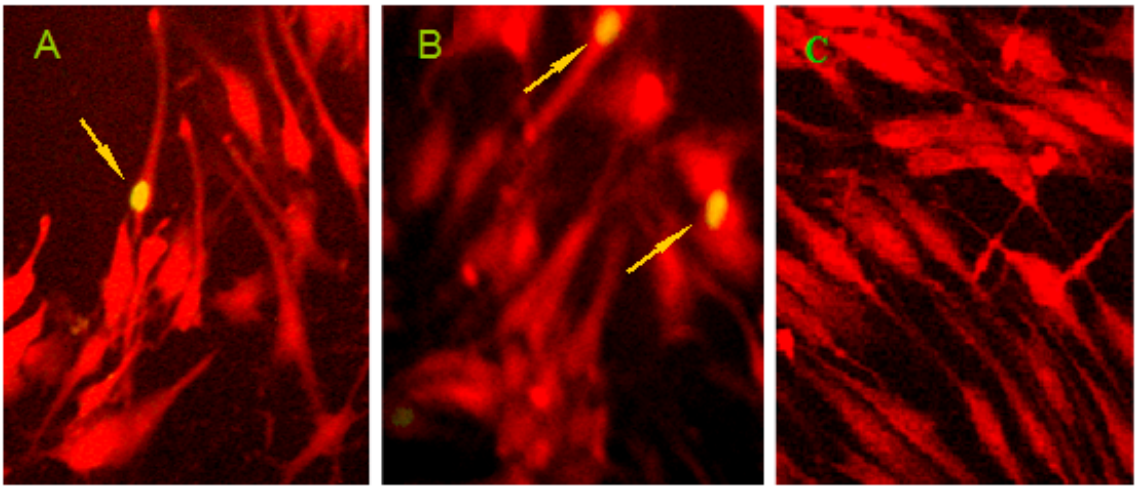


Figure 2. Shell vial culture for HCMV



A: Positive shell vial culture of urine sample immunofluorescence assay (×250) B: Positive shell vial culture of saliva sample C: Negative shell vial culture of urine sample Indirect

25% of all positive cases respectively. Abnormal liver function test with elevated serum γ-glutamyltransferase was found in 75% and conjugated hyperbilirubinemia in 62.5% of HCMV positive cases.

Discussion

Primary and recurrent maternal infections are associated with occurrence of congenital infections but perinatal infections are linked to high rates of maternal seropositivity and breastfeeding as lactating mothers shed HCMV in breast milk [7-9]. However, the risk of congenital and perinatal infections increases with prematurity and a significant proportion of babies subsequently develop neurological sequelae [10,11]. Therefore, early identification of congenital or perinatal HCMV infection, particularly in the NICU, is indicated to ensure adequate treatment and follow-up [12]. This is the first study assessing the occurrence of congenital and perinatal HCMV in Bahrain. Although there is a high HCMV seropositivity among pregnant women in the Arabian region [4,13,14], this is the first prospective study to investigate NICU congenital and perinatal HCMV rates as well as viral shedding in lactating women.

In Bahrain, HCMV screening is not performed as a part of the routine microbiological workup of neonatal sepsis and is only requested when HCMV infection is suspected. In addition, the diagnosis is done by serological method and confirmed by pp65 antigenaemia test. Extremely premature infants who have immature immune systems might be missed by these two methods. For the first time, genome amplification and shell vial culture techniques were used to detect HCMV infection in our setting. Our findings indicate good correlation between both methods for detection of HCMV in urine and saliva. However, the PCR amplification procedure was found to be faster and more robust with the advantage that samples can be frozen and batched. In addition, the practical issues such as manipulation of cover slips and sample toxicity to the cell culture associated with the shell vial method were avoided.

The findings indicate a low rate of congenital HCMV infection which is reflective of the high maternal HCMV IgG seropositivity. Comparisons with other countries in our region cannot be carried out due to lack of data. Congenital and perinatal HCMV infections in neonatal ICUs in other countries have shown variable prevalence rates ranging from 6.8% to 16%, with this variation being attributable to different maternal seroprevalence and gestational age of the study population [12,15-17]. Thus, compared to reports from other parts of the world, inclusive of those done in similar settings of HCMV maternal seropositivity, the rate of congenital HCMV in the NICUs reported here is on the lower end of the spectrum.

Hamprecht and colleagues [18] assessed the transmission rate and clinical significance of HCMV transmission in breast milk-fed premature babies. In their study, HCMV DNA was detected in breast milk samples in 96% of seropositive mothers, and 38% of their breastfed premature newborns acquired perinatal infections. Similarly, an earlier report by Vochem *et al*. [19] demonstrated that 85% of seropositive mothers shed HCMV DNA in breast milk and the transmission rate for their breastfed newborns was 59%. In our study, although the maternal HCMV (IgG) seropositivity rate was 100%, HCMV DNA detection in breast milk was 3.8% and 22.5% during the first 10 days and during 4 to 6 weeks postpartum respectively. Thus, while there is a relatively low prevalence of HCMV shedding in breast milk in our setting, the clinical significance of transmission via breast milk in mothers shedding the virus remains demonstrable as almost half of the babies who had HCMV infected breast milk developed perinatal infections. In addition, all of these were preterm babies with high risk of mortality and morbidity. Indeed, in 2004, anecdotal data from the NICU at SMC showed that 25% of fatalities among admitted preterm babies had sepsis-like disease of unknown aetiology. Based on the findings of high perinatal transmission, we speculate that HCMV, which is not routinely tested for, might be one of the causes of sepsis-like diseases. This finding underscores the need for an increased level of awareness of the risk of perinatal HCMV infection, particularly in NICUs in settings of high maternal seropositivity.

Cytomegalovirus infection is commonly associated with congenital abnormalities and intrauterine growth retardation (IUGR) [20]. Jaundice, sepsis, and respiratory distress syndrome were common clinical findings in our HCMV positive cohort with 25% having IUGR. However, the IUGR percentage was not higher in infected newborns compared to non-infected newborns (data not shown) although this is most likely related to the fact that non-infected babies in the NICU were most likely having other associated fetal or maternal diseases causing IUGR.

In conclusion, our findings indicate that both congenital HCMV infection and acquisition of HCMV during the perinatal period via breastfeeding occurs in our setting and should be considered in premature, low birth weight or symptomatic newborns. We recommend neonatal HCMV screening to detect infection during the first few postnatal weeks, especially for babies admitted into the NICU. This should be performed using rapid and sensitive methods such as PCR amplification or shell vial culture.

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References

1. Ornoy A and Tenenbaum A (2006) Pregnancy outcome following infections by coxsackie, echo, measles, mumps, hepatitis, polio and encephalitis viruses. Reprod Toxicol 21: 446-457.
2. Yinon Y, Farine D, Yudin MH (2010) Screening, diagnosis, and management of cytomegalovirus infection in pregnancy. Obstet Gynecol Surv 65: 736-743.
3. Mosca F and Pugni L (2007) Cytomegalovirus infection: the state of the art. J Chemother 19: 46-48.
4. Yousif AA, Wallace MR, Baig BH, Rajab K (1991) Prenatal serologic screening in Bahrain. Scand J Infect Dis 23: 781-783.
5. Distefano AL, Alonso A, Martin F, Pardon F (2004) Human cytomegalovirus: detection of congenital and perinatal infection in Argentina. BMC Pediatr 4: 11.
6. Kulkarni A, Westmoreland D, Fox JD (2001) Molecular-based strategies for assessment of CMV infection and disease in immunosuppressed transplant recipients. Clin Microbiol Infect 7: 179-186.
7. Kurath S, Halwachs-Baumann G, Muller W, Resch B (2010) Transmission of cytomegalovirus via breast milk to the prematurely born infant: a systematic review. Clin Microbiol Infect 16: 1172-1178.
8. Kurath S and Resch B (2010) Cytomegalovirus and transmission via breast milk: how to support breast milk to premature infants and prevent severe infection? Pediatr Infect Dis J 29: 680-681.
9. Schleiss MR (2006) Role of breast milk in acquisition of cytomegalovirus infection: recent advances. Curr Opin Pediatr 18: 48-52.
10. Hamprecht K, Maschmann J, Jahn G, Poets CF, Goelz R (2008) Cytomegalovirus transmission to preterm infants during lactation. J Clin Virol 41: 198-205.
11. Luck S and Sharland M (2009) Postnatal cytomegalovirus: innocent bystander or hidden problem? Arch Dis Child Fetal Neonatal Ed 94: F58-64.
12. Nagy A, Endreffy E, Streitman K, Pinter S, Pusztai R (2004) Incidence and outcome of congenital cytomegalovirus infection in selected groups of preterm and full-term neonates under intensive care. In Vivo 18: 819-823.
13. Al-Nakib W, Ibrahim ME, Hathout H, Moussa MA, Deverajan LV, Thorburn H, Yousof AM (1983) Seroepidemiology of viral and toxoplasmal infections during pregnancy among Arab women of child-bearing age in Kuwait. Int J Epidemiol 12: 220-223.
14. Ghazi HO, Telmesani AM, Mahomed MF (2002) TORCH agents in pregnant Saudi women. Med Princ Pract 11: 180-182.
15. Oda K, Oki S, Tsumura N, Nakao M, Motohiro T, Kato H (1995) Detection of cytomegalovirus DNA in urine from newborns in NICU using a polymerase chain reaction. Kurume Med J 42: 39-44.
16. Panhani S and Heinonen KM (1994) Screening for congenital cytomegalovirus infection among preterm infants born before the 34th gestational week in Finland. Scand J Infect Dis 26: 375-378.
17. Santos DV, Souza MM, Goncalves SH, Cotta AC, Melo LA, Andrade GM, Brasileiro-Filho G (2000) Congenital cytomegalovirus infection in a neonatal intensive care unit in brazil evaluated by PCR and association with perinatal aspects. Rev Inst Med Trop Sao Paulo 42: 129-132.
18. Hamprecht K, Maschmann J, Vochem M, Dietz K, Speer CP, Jahn G (2001) Epidemiology of transmission of cytomegalovirus from mother to preterm infant by breastfeeding. Lancet 357: 513-518.
19. Vochem M, Hamprecht K, Jahn G, Speer CP (1998) Transmission of cytomegalovirus to preterm infants through breast milk. Pediatr Infect Dis J 17: 53-58.
20. Ornoy A, Diav-Citrin O (2006) Fetal effects of primary and secondary cytomegalovirus infection in pregnancy. Reprod Toxicol 21: 399-409.

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