



Short communication

Real-time PCR versus viral culture on urine as a gold standard in the diagnosis of congenital cytomegalovirus infection

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ABSTRACT

Background: Cytomegalovirus (CMV) infection is the most common cause of congenital infection. Whereas CMV PCR has replaced viral culture and antigen detection in immunocompromised patients because of higher sensitivity, viral culture of neonatal urine is still referred to as the gold standard in the diagnosis of congenital CMV infection.

Objective: To compare real-time CMV PCR with shell vial culture on urine in the diagnosis of congenital CMV, in a multicenter design.

Study design: A series of neonatal urines ($n = 340$), received for congenital CMV diagnostics and routinely assessed with shell vial CMV culture, was retrospectively tested by real-time CMV PCR.

Results: The proportion of newborns found to be congenitally infected by real-time CMV PCR was 8.2% (28/340, 95%CI 5.6–11.8%), and 7.4% (25/340, 95%CI 4.9–10.8%) by rapid culture. When considering rapid culture as reference, real-time PCR was highly sensitive (100%), whereas sensitivity of rapid culture was 89.3% when considering real-time PCR as reference.

Conclusions: Our results, supported by analytical and clinical data on CMV DNA detection in neonatal urine, suggest enhanced sensitivity of recent PCR techniques when compared to viral culture. There is considerable rationale to favor real-time CMV PCR as a gold standard in the diagnosis of congenital CMV infection. A large-scale study combining both laboratory and clinical data is required to determine the exact time frame for sampling of neonatal urine when using real-time PCR.

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1. Background

Cytomegalovirus (CMV) infection is the most common cause of congenital infection and a leading cause of non-genetic sensorineural hearing loss.^{1–3} For decades, the gold standard in the diagnosis of congenital CMV infection has been viral culture of urine, sampled within the first 2 or 3 weeks of life.^{4–6} After this period, CMV present in urine may be due to postnatally acquired infection. Meanwhile, PCR assays have been optimized by with improved extraction and amplification techniques (e.g. real-time detection and internal controls for PCR inhibition), resulting in highly sensitive and specific assays. CMV DNA detection has become a routine diagnostic tool at many centers thanks to its rapid, reproducible, automated and quantitative nature.^{7,8} Experiments with dilution

series have shown that the analytical sensitivity of CMV PCR on urine is approximately 100 times higher than both traditional tube and shell vial culture.⁹ In immunocompromised patients, CMV PCR has replaced CMV blood culture and pp65 antigen detection because of the higher sensitivity.^{10–12} Furthermore, the clinical sensitivity of CMV PCR on urine of kidney and liver transplant patients is higher than viral culture,^{13–15} with CMV DNA loads in urine being predictive of CMV disease.^{11,16,17} Strikingly, in recent guidelines and reviews on congenital CMV, viral culture of neonatal urine remains referred to as the gold standard for confirmatory diagnosis, while CMV PCR is mentioned as plausible alternative more frequently.^{18–20}

2. Objective

The aim of this study was to compare real-time CMV PCR with shell vial culture on urine in the diagnosis of congenital CMV, in a multicenter design.

Abbreviations: CMV, cytomegalovirus.

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Table 1
Comparison of internally controlled real-time PCR with shell vial culture of urine samples from newborns ($n = 340$) in the diagnosis of congenital CMV infection.

| | Real-time CMV PCR | | |
|------------------------------|-------------------|--------------------|-----------|
| | Positive | Negative | Total |
| Rapid CMV culture | | | |
| Positive | 25 | 0 | 25 (7.4%) |
| Negative | 3 | 312 | 315 |
| Total | 28 (8.2%) | 312 | 340 |
| Reference: real-time PCR | | | |
| Sensitivity culture (95% CI) | | 89.3% (70.6–97.2%) | |
| Specificity culture (95% CI) | | 100% (98.5–100%) | |
| Reference: rapid CMV culture | | | |
| Sensitivity PCR (95% CI) | | 100% (83.4–100%) | |
| Specificity PCR (95% CI) | | 99.1% (97.0–99.8%) | |
| Kappa (95% CI) | | 0.94 (0.87–1) | |

3. Study design

3.1. Urine samples

A series of neonatal urines, sampled within the first 3 weeks after birth, received for congenital CMV diagnostics and routinely assessed with shell vial CMV culture,²¹ was retrospectively tested by real-time CMV PCR.^{22–24} All CMV culture positive samples ($n = 25$) and a large random selection of CMV culture negative urine samples ($n = 315$) dating from 2001 to 2011, were included in the analysis, irrespective of clinical characteristics of the newborns. All diagnostic urine samples were stored at -80°C in the Dutch participating laboratories until tested by real-time PCR at that specific site (Leiden University Medical Center (LUMC), $n = 61$ urines), Erasmus Medical Center Rotterdam (Erasmus MC), $n = 199$ urines), and Academic Medical Center Amsterdam (AMC), $n = 80$ urines). Because of ethical reasons, retrospective testing was performed anonymously.

3.2. Viral culture and real-time PCR

Shell vial culture and CMV DNA extraction followed by amplification using seal herpesvirus (PhHV-1) as internal PCR control were performed as described previously.^{21–24} In short, extraction was performed on the MagnaPure LC station using the Total Nucleic Acid Isolation Kit – High Performance Kit (both Roche Diagnostics, Almere, The Netherlands) (all sites), and the PCR was carried out using a CFX96 TM real-time PCR detection system (BioRad, Veenendaal, The Netherlands) (LUMC)/a LightCycler480 PCR system (Roche Diagnostics, Almere, The Netherlands) (Erasmus MC, AMC). Amplified was a 126-bp fragment of the CMV immediate-early antigen region (LUMC, AMC)/a 133-bp fragment of the CMV DNA polymerase gene (Erasmus MC).

3.3. Statistical analysis

Sensitivity and specificity were calculated with both rapid CMV culture and real-time CMV PCR as reference. Kappa was calculated to assess test agreement.

4. Results

In total 340 urine samples of newborns ≤ 3 weeks of age were included in the comparison and were retrospectively tested with CMV real-time PCR (Table 1). The proportion of newborns found to be congenitally infected by rapid culture was 7.4% (25/340, 95%CI 4.9–10.8%), and 8.2% (28/340, 95%CI 5.6–11.8%) by real-time CMV PCR. All culture positive samples were detected by CMV PCR. In contrast, three urine samples were detected by real-time PCR that

Table 2
CMV DNA load and time of sampling of the urines with discrepant test results.

| Rapid CMV culture | Real-time CMV PCR (copies CMV DNA/ml) | Time of urine sampling (days after birth) |
|-------------------|---------------------------------------|---|
| Negative | Positive (24,000) | 17 |
| Negative | Positive (64,000) | 17 |
| Negative | Positive (210,000) | 10 |

were negative in rapid CMV culture. When considering rapid culture as reference, real-time PCR was highly sensitive (100%) and specific (99.1%). Sensitivity of rapid culture was 89.3% when considering real-time PCR as reference. The CMV DNA load of the three samples with discrepant results (median 64,000 copies/ml, range 24,000–210,000 copies/ml, Table 2) was lower than the load of the 25 culture-positive samples (median 260,000 copies/ml, range 4400–95,000,000 copies/ml). These three urines were sampled at day 10, 17 and 17 of age, respectively, whereas the median time of sampling of the 25 culture-positive samples was 3 days (range 0–11 days). Additional testing of the discrepant samples by repeated extraction and amplification of a different target (gB) gene (at a different participating center) yielded confirmatory positive PCR results.

5. Discussion

In our multicenter comparison, CMV was more frequently detected in urine samples of newborns by real-time PCR than by rapid culture, which is still referred to as the reference method for diagnosing congenital CMV infection. These discrepant test results theoretically can be attributed to either false negative viral culture results, or false positive real-time PCR results. False negative viral culture results have been described both in experimental setting⁹ and in clinical setting, testing urine samples of (immunocompromised) patients.^{13–15,24} Loss of viable CMV particles implicated in false negative culture results may be caused by transport at room temperature²⁵ and antiviral therapy. In our analysis, two of the three discrepant samples had transport times of >1 day. Concerning potential false positive PCR results, the use of real-time PCR procedures (which are less prone to contamination than nested procedures), the use of negative controls and confirmatory testing of the discrepant samples, render false positive CMV DNA detection in our study highly unlikely. Hence, false negative results in the viral culture assays constitute the most likely explanation for the discrepant test results in our study.

Previous studies comparing viral culture with CMV PCR on urine as initial or screening assay in the diagnosis of congenital CMV were reviewed, and sensitivity and specificity was calculated with both rapid CMV culture and real-time CMV PCR as reference (Table 3). Assuming PCR as reference, sensitivity of viral culture ranged from 61.5% to 100%. Negative CMV PCR results of culture positive urine samples were only described in earlier studies in which per report several gel-based PCR assays were compared while optimizing sensitivity.^{26,27} Internal controls for potential PCR inhibiting components present in urine^{28,29} were lacking in these studies.

Two of the three samples with discrepant test results in our study were taken 17 days after birth, and we cannot exclude that these were derived from postnatally acquired CMV infections. Postnatal CMV infection commonly occurs, because of frequent acquisition of CMV in the birth canal or from breast milk.⁶ The restriction of samples taken within the first 3 weeks of life is considered safe to demonstrate congenitally acquired CMV infection.^{5,6} However, literature contains mixed references to this time frame,^{4,5,18,20} which is based on viral culture techniques and might not necessarily be identical for more sensitive diagnostic methods. Previous data have shown that CMV replicates with a

Table 3
Reports with data on both viral culture and CMV PCR on urine as initial or screening assay for diagnosing congenital CMV. Sensitivity and specificity of both viral CMV culture and CMV PCR were (re-)calculated with as reference method viral culture and CMV PCR, respectively. IC: inhibition control, cCMV: congenital CMV.

| Reference [publication year] | Newborns included (age at sampling) | PCR method | Culture method ^a | Proportion CMV positive ^b | Reference: CMV PCR (%) | | Reference: viral culture (%) | |
|------------------------------|---|------------------------------------|-----------------------------|--------------------------------------|------------------------|---------------------|------------------------------|-----------------|
| | | | | | Sensitivity culture | Specificity culture | Sensitivity PCR | Specificity PCR |
| Present study | Suspected cCMV (<3 weeks) | Real-time, IC | Shell vial | 8.2% (28/340) | 89.3 | 100 | 100 | 99.1 |
| 31 [2011] | Suspected cCMV (<3 weeks ^c) | Real-time, ^d IC | Shell vial | 3.8% (10/260) | 100 | 100 | 100 | 100 |
| 32 [2010] | Screened newborns (<2 weeks) | Real-time, IC | Shell vial | 3.3% (3/91) | 66.7 | 100 | 100 | 98.9 |
| 33 [2005] | Suspected cCMV (<3 weeks ^c) | Nested, ^d IC, gel-based | Shell vial | 12.6% (32/254) | 100 | 100 | 100 | 100 |
| 34 [2001] | Screened newborns (<3 weeks) | Multiplex, gel-based | Tube | 2.1% (7/332) | 100 | 100 | 100 | 100 |
| 35 [2000] | Suspected cCMV (<2 weeks) | Real-time | Shell vial | 75.0% (9/12) | 77.8 | 100 | 100 | 60 |
| 27 [1996] | Screened newborns (<3 days) | Gel-based ^e | Tube | 1.8% (18/1000) | 100 | 99.9 | 94.4 | 100 |
| 26 [1994] | Suspected cCMV (majority), postnatal, immunocompromised | Gel-based ^e | Shell vial | 36.9% (52/141) | 61.5 | 97.8 | 94.1 | 81.3 |
| 36 [1988] | Screened newborns | Dot blot-based ^e | Tube | 62.0% (44/71) | 100 | 100 | 100 | 100 |

^a Shell vial culture included staining with CMV (early) antigen-specific MAbs.

^b Based on the number of PCR positive samples, with the exception of ²⁷.

^c 241 out of 254 newborns were sampled <3 weeks of age.

^d Pool testing.

^e Multiple PCR assays per study; data from the optimized PCR with improved sensitivity were used in the calculations.

doubling time of approximately one day.³⁰ Assuming that the analytical sensitivity of CMV PCR on urine is 100 times more sensitive than viral culture,⁹ PCR could theoretically detect CMV 6.6 days (100 log₂) earlier than viral culture, hence on day 14 postpartum compared to day 21 when using viral culture. Unfortunately, partially because of ethical reasons, neither clinical data nor other materials (saliva, (dried) blood) could be retrieved from these three newborns to discriminate congenital and postnatal infection. Data from studies combining clinical data with real-time CMV PCR results should address the distinction between congenitally and postnatally acquired CMV infection.

In conclusion, our results are supported by with analytical and clinical data on CMV DNA detection in neonatal urine and suggest enhanced sensitivity of recent PCR techniques. There is considerable rationale to favor real-time CMV PCR as a gold standard in the diagnosis of congenital CMV infection. A large-scale study combining laboratory and clinical data is required to determine the exact time frame for sampling of neonatal urine when using real-time PCR.

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Competing interests

No conflict of interest.

Ethical approval

Ethical approval was not required.

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