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Laboratory diagnosis of congenital CMV infection in newborns: Impact of pre-analytic factors

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ABSTRACT

Background: To identify infants with congenital cytomegalovirus (cCMV) saliva polymerase chain reaction (PCR) is an ideal screening method. However, there are only few data on the influence of pre-analytic factors on the analytical sensitivity of the CMV PCR.

Objectives: This study aimed to evaluate the performance of different swabbing materials, transport time and initial virus concentration regarding to the efficacy of recovery of CMV-DNA.

Study design: Two CMV suspensions containing a high or low concentration of the laboratory strain AD 169 were prepared as test samples. Sampling was simulated by immersion of different swabs in these CMV suspensions and storing the swabs dry or in specified transport media. Transport conditions were modeled by storing the samples for defined time periods prior to DNA extraction and quantitative PCR analyses. Parallel analyses in two different laboratories allowed determination of lab to lab consistency.

Results: The duration of storage under the conditions analysed did not have a major effect on the recovery efficiency for the swabbing materials tested. With exception of flocked dry swabs, all tested swabbing materials demonstrated good recovery of CMV DNA. The flocked swab/eNAT system showed the best overall performance.

Conclusions: All tested swabbing materials (with exception of the flocked dry swabs) seem to be well suited for recovery of CMV DNA and appropriate for use for the diagnosis of cCMV infection in symptomatic cases and in general cCMV screening programs of newborns.

1. Background

Congenital cytomegalovirus (cCMV) infection is common, with an estimated birth prevalence of 0.2% in US, Canada, Australia, and Western Europe [1], while limited studies in developing countries (Latin America, Africa, Asia) report prevalences ranging from 0.6 to 6.1% [2]. The vast majority of newborns with cCMV infection are asymptomatic, approximately 10% however show clinical, laboratory or imaging abnormalities. Long term sequelae (e.g. late onset sensorineural hearing loss (SNHL), visual impairment, cognitive and motor deficits) occur in 40–60% of infants with and in 10–15% of infants without symptoms at birth [3]. Without a general screening, the majority of cCMV infections is not diagnosed at birth and affected newborns do not receive the necessary follow-up for timely detection of possible sequelae (mostly SNHL in infants with asymptomatic cCMV) that would allow early interventions (e.g. hearing aids, speech therapy).

A large-scale study on about 35,000 newborns has shown that saliva polymerase chain reaction (PCR) is a sensitive and specific method to identify infants with cCMV infection [4]. This method is more convenient than obtaining urine (urine culture has been gold standard) and amenable to high-throughput assays. However, the influence of pre-analytic factors on the analytical sensitivity of the CMV PCR has not been reported. Therefore, the current study aimed to evaluate the performance of different swabbing materials, transport time and initial virus concentration with regard to the efficacy of recovery of CMV DNA. In future, this should improve the accuracy of quantitative

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Table 1
Swabs tested in this study.

Swab No.	Swab type (medium)	Volume	Remarks
1	Flocked[a] (dry)	–	For elution, 1 ml PBS was added and swabs were incubated for 10 min prior to further processing.
2	Traditional[b] (dry)	–	For elution, 1 ml PBS was added and swabs were incubated for 10 min prior to further processing.
3	Flocked[a] (VTM[c])	1 ml	
4	Traditional[b] (VTM[c])	1 ml	
5	Flocked (UTM[d])	1 ml	
6	Flocked (eNAT[e])	1 ml	

[a] Flocked swab (FLOQSwab™; Copan Italia, Brescia, Italy; order number: 502CS01).
[b] Rayon tipped swab (CLASSIQSwabs™; Copan Italia, Brescia, Italy; order number: 155C).
[c] The in-house VTM (Labor Enders, Stuttgart, Germany) consists of veal infusion broth supplemented with bovine serum albumin and D-sucrose. Phenol red is used as a pH-indicator. Gentamicin, vancomycin and amphothericin B are added to inhibit contamination.
[d] UTM™ with corresponding flocked swab (Copan Italia, Bresca, Italy; order number: 360C).
[e] eNat™ with corresponding flocked swab (Copan Italia, Bresca, Italy; order number: 608CS01R).

assessment of CMV DNA in swab material and facilitate investigations on the predictive value of CMV viral load of swab samples on the risk of late-onset cCMV disease.

2. Study design

2.1. Preparation of test samples

Two virus suspensions (suspension 1 and 2) containing approximately 120,000 and 15,000 international units (IU)/ml of DNA of the laboratory strain CMV-AD 169, respectively were prepared as test samples. Phosphate buffered saline (PBS) was used as diluent because it proved suitable in a previous feasibility study (data not shown). The suspensions were used immediately after preparation.

2.2. Test sample collection and storage

Different swabbing materials (Table 1) were immersed at the diagnostic laboratory 1 (Institute of Medical Virology, University Hospital Frankfurt, Germany) with a rotating movement in suspension 1 and 2 for 4–6 seconds. Subsequently the swabs were stored dry or immersed in 1 ml storage media as indicated in Table 1. The transport conditions were simulated by storing the samples afterwards at room temperature for 2 h, 24 h or 8 days. One additional set of samples was sent at ambient temperature to a second independent diagnostic laboratory (Institute of Clinical and Molecular Virology, University Hospital Erlangen, Germany). These samples were stored at room temperature before shipment and from arrival until DNA extraction on day 8 after sampling. All experiments were done in triplicates.

Dry swabs (Table 1, swab no. 1 & 2) were eluted in PBS prior to further processing. In a preliminary study PBS proved to perform better than distilled water and was equivalent to the other transport media given in Table 1 (viral transport medium [VTM], universal transport medium [UTM], eNAT) when used as diluent (data not shown).

2.3. DNA extraction and quantitative PCR testing

Dry swabs were incubated in 1 ml of PBS for 10 min. All swabs were vortexed for 10 s at maximum speed. For nucleic acid extraction in diagnostic laboratory 1, 500 μl of each sample was eluted in a final volume of 130 μl using the QIAsymphony (Qiagen GmbH, Hilden, Germany) together with the DSP virus/pathogen midi kit (Qiagen GmbH, Hilden, Germany). After extraction the nucleic acid was stored at −80 °C until PCR testing was performed. For the assessment of CMV DNA load, an internally controlled CMV real-time PCR using the TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, Darmstadt, Germany) and the ABI7500 sequence detection system (Thermo Fisher Scientific, Darmstadt, Germany) was performed. 10 μl of the extracted DNA were amplified with primers and probe targeting the UL89 gene of CMV. As internal control, a defined number of cell culture supernatant-derived murine CMV (mCMV; strain Smith; ATCC VR-1399) virions were added [5]. Based on calibration of an in house CMV DNA standard to the CMV WHO Standard, viral load values were expressed as IU/ml virus suspension with a lower limit of detection of 200 IU/ml.

The CMV PCR at diagnostic laboratory 2 was performed as follows: The DNA was extracted from 200μl of each sample and eluted in a final volume of 60 μl using the QIAsymphony® DSP Virus/Pathogen Mini Kit on a QIAsymphony automated extraction machine according to the manufacturer´s instructions (Qiagen GmbH, Hilden, Germany). After extraction the nucleic acid was stored at −80 °C until PCR testing. As positive control, CMV containing cell culture supernatant was used, distilled water served as negative control (Sigma, Taufkirchen, Germany). Before extraction a defined number of Herpesvirus saimiri (HVS) DNA copies was added to each sample acting as control for extraction and PCR inhibition. PCR was performed using the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Darmstadt, Germany) and the ABI7500 sequence detection system (Thermo Fisher Scientific, Darmstadt, Germany). 10 μl of the extracted DNA were amplified with primers and probe targeting the IE-1 gene of CMV as described previously [6]. Quantification of CMV DNA was performed using a standard curve with known amounts of an IE-1 CMV PCR product generated from a CMV positive patient sample. The IU of the CMV standard DNA had been calibrated using the WHO International Standard (NIBSC code: 09/162) allowing to express all results as IUs with a lower limit detection of 250 IU/ml virus suspension.

In both laboratories, all swab samples were analysed by PCR in one test run, which also included aliquots of virus suspensions 1 and 2 stored for the same duration as the swabs. Both laboratories follow strict quality assurance principles (including external quality control programs) and are accredited by the national accreditation body for the Federal Republic of Germany (DAkkS) according to DIN EN ISO 15189 for the diagnostic laboratory and the CMV DNA analysis.

2.4. Predicting the amount of virus absorbed

To quantify the volume of the virus suspensions the different swab types could absorb, a tube containing 1 ml of distilled water was initially weighed and each swab type was immersed in the volume under rotating movement for 4–6 seconds. After removing the swabs the tubes were weighed again and the volume absorbed by each swab was calculated from the weight differences of the tubes based on the density of water of 1. Measurements were done in triplicates. With the knowledge of the viral concentrations of the suspensions used in the experiment, the hypothetic amount of absorbed viral material could be calculated.

Table 2
Absorbed fluid volume by swab type and hypothetic concentration of absorbed viral material from suspension 1 and 2 (original viral load 120,000 and 15,000 IU/ml in laboratory 1 as well as 41,500 and 5000 IU/ml in laboratory 2 respectively):

Swab No.	Swab type	Measured volume (ml) as mean (standard deviation)	Hypothetic virus load (IU/ml) laboratory 1		Hypothetic virus load (IU/ml) laboratory 2	
			suspension 1	suspension 2	suspension 1	suspension 2
1, 3	Flocked	0.101 (± 0.003)	12,100	1,500	4,200	500
2, 4	Traditional	0.072 (± 0.004)	8,600	1,100	3,000	360
5	Flocked (UTM version)	0.073 (± 0.002)	8,800	1,100	3,000	370
6	Flocked (eNAT version)	0.101 (± 0.007)	12,100	1,500	4,200	500

2.5. Data analysis

The collected data were entered and analyzed with Microsoft Excel® (MS Office, 2010; Microsoft Corporation, USA) and GraphPad Prism® 6.01 (GraphPad Software, USA). Considering the normal distribution of the generated data parametric tests for independent samples were used. For comparison of the different swab types (see Table 1) the one-way analysis of variance (ANOVA) was performed at first. In the case of $p < 0.05$, differences between two swab types each were compared using the Tukey´s Multiple Comparison Test. Level of significance α was defined as less than 0.05 ($p < 0.05$). Apart from that, results are shown as mean ± SEM.

3. Results

To explore potential differences in the volume the different swabs absorb, the loss of weight of tubes filled with water after soaking the different swabs under defined conditions was determined. This revealed that the different swabs absorbed between approximately 0.07 and 0.1 ml (Table 2). Based on the absorbed volume and the CMV DNA concentration of two virus suspensions, the hypothetical viral load of each swab was calculated. In the absence of a joint standard, viral load determinations may differ several-fold between laboratories. Since we aimed to compare recovery efficiencies for the different swabs between two different laboratories, the CMV DNA concentrations of aliquots of the same two virus suspensions were determined independently in both laboratories. This revealed 3-fold lower CMV DNA concentrations for both virus suspensions in laboratory 2. Therefore, different hypothetical viral loads were calculated for each laboratory.

The impact of the different swab types and the duration of storage on the recovery efficiency of CMV DNA was determined for virus suspension 1 (Fig. 1a) and 2 (Fig. 1b). Comparing the CMV DNA concentration determined for each of the experimental conditions to the hypothetical values provided a measure of efficiency of recovery that also allowed comparison between the two laboratories.

Recovered virus concentrations of the particular swab types were similar at different time points as illustrated by Fig. 1. Thus, CMV DNA recovery of the different swabbing materials seems to be independent of the duration of storage reflecting the transport time. However, the swab and transport medium seemed to affect recovery efficiency to a larger extent. Swab no. 1 revealed a significantly lower recovery than the same swab used with VTM (swab no. 3) at each time point (Fig. 1a, b). In contrast, there is obviously no such difference between the second dry swab (swab no. 2) and the same swab soaked with VTM (swab no. 4). The reproducibility within the same swabs was predominantly excellent.

To quantitatively assess potential differences in CMV DNA recovery between different sampling procedures, the mean CMV DNA recovery for both virus suspensions at all time points in both laboratories was calculated for each swab type (Fig. 2). Swabs no. 3 and 6 showed the highest mean recovery of CMV DNA. Statistical analysis of CMV DNA recovery rates of the swabs analysed (no. 1-6) using one-way ANOVA showed significant differences ($p < 0.0001$). Results of the group to group comparisons with the Tukey´s Multiple Comparison Test are shown in Fig. 2. With swab no. 1 the lowest recovery rate of all swab types could be detected (40% of hypothetical value, Fig. 2) – this amount is significantly lower than in three other swab types (Fig. 2). Furthermore, flocked swab with eNat medium (swab no. 6) had the highest mean recovery rate (160% of hypothetical value, Fig. 2) that differed significantly from the recovery rates from swab 1, 4 and 5 (Fig. 2).

4. Discussion

Congenital CMV infection is the most common non-genetic cause of SNHL in childhood. The fact that most congenitally infected infants are asymptomatic at birth underlines the need for postnatal CMV screening, as early detection is essential for directed care [7]. The ideal screening method should be rapid, sensitive, cost effective and amenable to high-throughput testing. Saliva is easy to collect with swabs and PCR methods are widely used. Reports in the past indicate that this approach has good analytical performance and clinical sensitivity and may be able to fulfill the criteria for qualitative screening purposes [4]. Nevertheless the rate of false positive quantitative real time (qrt) PCR results from saliva compared to confirmation with PCR from urine was reported discordantly ranging from 1.2% [8] to 16% [9]. Importantly, contamination of saliva with CMV after breastfeeding leading to false positive PCR results seems to be less critical than previously thought [10].

One of the caveats of an universal CMV screening approach is the identification of congenital CMV infections in newborns that are asymptomatic at birth and will never develop symptoms. Unnecessary parental worries by the threating diagnosis of cCMV infection and an intensified follow-up could be avoided if a prognostic marker was available. The viral load in the saliva could be such a prognostic marker, but exploring this would require reliable quantitative determination of viral load levels in saliva samples. Therefore, the current study explored a number of preanalytical factors affecting CMV viral load measurement by different swabs and storage conditions.

Our study demonstrates that the duration of storage under the conditions analysed did not have a major effect on the recovery efficiency for any of the swabs (Fig. 1). However, up to 4-fold differences in the recovery efficiencies were observed between the different swabs tested (Fig. 2). Using the flocked swab with the eNAT-medium (swab no. 6) or VTM (swab no. 3), the highest amounts of CMV DNA could be recovered (Fig. 2). In contrast to the study done by [11], the traditional swab without transport medium (swab no. 2) performed well in our study. It recovered comparable amounts of virus DNA at different time points like the traditional swab with VTM (swab no. 4) and the flocked swab with UTM (swab no. 5). The difference could be due to the fact that in the study by Goshen et al. distilled water was used as a diluent of dry swabs prior to extraction. In an initial feasibility study we observed that distilled water proved not to be suitable as diluent for the dry swabs (data not shown). For some of the preparations the recovered virus concentration after 8 days was slightly higher in comparison to the recovery after two hours. Maybe this is due to the fact that after a

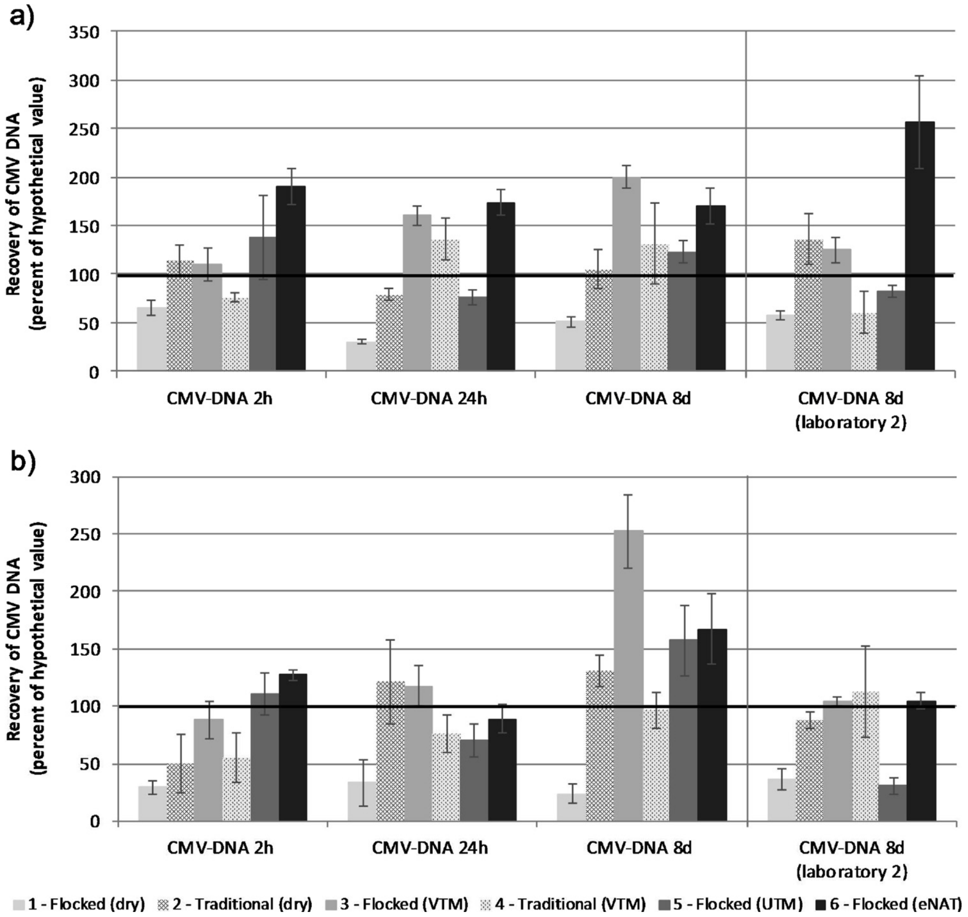

Fig. 1. Comparison of the recovered CMV DNA (mean value ± SEM) from various swab types soaked in suspension 1 (a) and suspension 2 (b) at different time points as percentage of the hypothetical viral load (marked by the black line). Shown are the results of three independent measurements of each swab type for each time point.

longer storage period of the swab in the UTM, more viral particles got released from the matrix of the swab. In our study, dry swabs were pre-incubated in PBS for 10 min (and vortexing for 10 s) before extraction of nucleic acids. Preliminary tests showed that this pre-incubation leads only to a slight improvement in CMV DNA extraction (data not shown). Therefore this step can be dropped where necessary to make the laboratory screening procedure even faster and with reduced hands-on time.

The amount of CMV DNA detected for the different swabs diverged approximately 3-fold (0.5 $\log_{10}$) between the two laboratories. This minor variation is most likely due to differences in the standards used. Substantial interlaboratory variability in quantitative CMV viral load values up to 2 $\log_{10}$ copies/ml for individual samples was already found in a multicenter study [12] and could be also observed in the national ring trial (organized by the German Society for Promotion of Quality Assurance in Medical Laboratories, INSTAND e.V.), where a deviation from the expected result by up to a factor of 6.3 are tolerated in order to pass the test. This indicates that the interlaboratory variation may be larger than the variations observed between the different swabs and storage conditions. However, since both variations may sum-up, both need to be controlled for establishment of quantitative assays of CMV viral load in saliva samples.

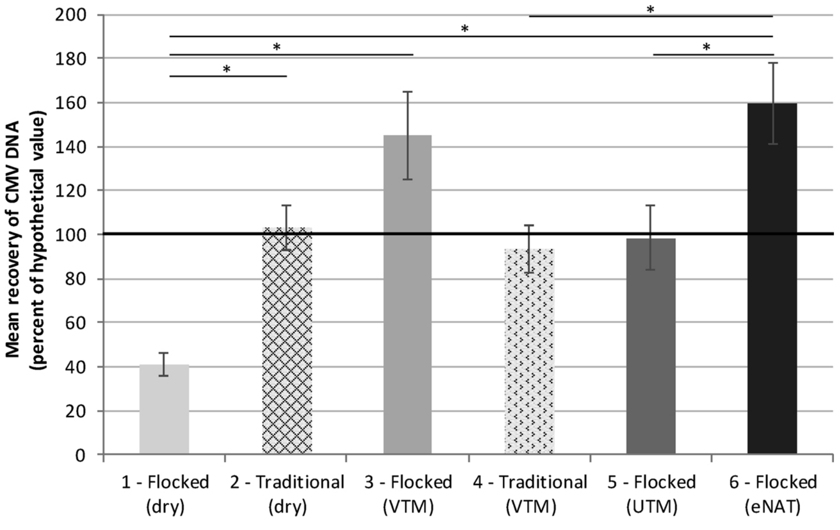

Fig. 2. The mean CMV DNA recovery efficiency (± SEM) was calculated for each swab from the mean recovery efficiency for all time points of both suspensions shown in Fig. 1a + b. The 100% recovery efficiency is marked by the bold line. All statistical significant differences between groups by Tukey´s multiple comparison test are indicated by the horizontal bars. (* $p < 0.05$).

To summarize: Flocked swabs with eNAT or VTM showed the best recovery rates of CMV DNA in our study. The commercial availability of eNAT system allows use of standardized reagents. However, all swabbing materials (with exception of dry swab no. 1) seem to be well suited for recovery of CMV DNA and seem appropriate for use in general cCMV screening of newborns. This is an important result regarding cost effectiveness of an implemented screening, because the flocked swab/eNAT system is currently the most expensive one.

Ethical approval

Not required.

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Competing interests

None declared.

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