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Human cytomegalovirus quantification in toddlers saliva from day care centers and emergency unit: A feasibility study

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ABSTRACT

Background: Cytomegalovirus (CMV) infection is the most important cause of congenital viral infection in developed countries. In utero transmission occurs at higher rates in seronegative women during primary infection, especially those in contact with young children in day-care centers (DCC). Nevertheless data on variability of CMV excretion among children in French DCCs are lacking, and are important for public health planning.
Objectives: Our main objective was to assess the feasibility of a salivary sample in DCCs in order to study CMV excretion among toddlers. Our secondary aims were to assess prevalence of CMV excretion in children attending Hospital Emergency Unit (EU) in comparison with various types of DCCs and to validate the analytical chain for collected specimens.
Study design: Excretion of CMV in saliva was quantified using a real-time PCR assay in children aged from 3 months to 6 years old in EU and in DCC, with gB, gH and gN genotypes determined in infected children. Salivary sampling was performed using small sponges placed into a DNA conservation medium. Socio cultural and medical information were collected from attending parents.
Results: A total of 625 children were included, with 256 from six DCCs and 369 from one EU. In DCCs, the acceptability of the procedure was 87.3% (95%CI 78.5–96.2) amongst parents and children, and in the EU, acceptability was higher at 97.6% (95%CI 95.5–98.9). CMV shedding overall prevalence was 21.7% (95%CI 17.6–26.2), with CMV shedding prevalence in DCCs of 51.9% (95%CI 22.8–81.1).
Conclusion: We validated the feasibility and acceptability of measuring CMV shedding in the saliva of French toddlers. The discrepancy between CMV infection rates in day care centers and in the general population (as sampled in the EU) indicates the need for a further study to determine risk factors and shedding levels in the DCC population.

1. Introduction

Cytomegalovirus (CMV) is the leading cause of viral congenital infection in the world [1]. Materno-fetal transmission occurs in 30–50% of primary infections, leading to birth defect, or late sequelae such as hearing loss, mental retardation and developmental disabilities. In developed countries nearly 50% of women of childbearing age are immune, based on IgG seropositivity [2,3]. Children in day care centers (DCCs) are known to be a major reservoir of virus [4,5]. Genotypes shed in saliva or urine circulate among toddlers, and can then be transmitted to seronegative pregnant women [6,7]. The factors responsible for differences in CMV shedding between toddlers have not been fully determined [7–9]. Using culture or qualitative PCR detection, previous studies were not able to quantify viral shedding in toddler's saliva or to study viral populations [6–8,10–12]. Finally, clinical aspects of CMV infection are

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well known in adults [13–15] or preterm infants [16,17], but less is known about infection of toddlers [18]. In particular, no clinical or shedding prevalence data are available for the French population from DCCs. One major practical difficulty is gaining access to the DCC for research and obtaining parental agreement for sampling of children. A further practical difficulty is obtaining reproducible and reliable sampling in this population.

2. Objectives

We performed a pilot study with the primary aim of assessing the feasibility of collecting salivary samples in DCCs. Our secondary aim was to characterize CMV shedding (prevalence, viral load, genotypes) in children attending the Emergency Unit (EU) of the Limoges teaching Hospital, and compare the results with those from different DCCs.

3. Study design

3.1. Study population

Two populations of children from 3 months to 6 years-old were studied: (1) children admitted to the EU of Limoges Hospital in southern France, recruited over three months, and (2) children who attended six DCCs that greatly varied in size and characteristics (Fig. 1) where samples were collected over a given week. Pediatricians gave the parents oral and written information, and a refusal notification to allow withdrawal at the time or later. A child refusing to participate was also considered as a refusal. For DCCs a video showing the objectives and conduct of the study was presented during a meeting, and available on the CMV reference center website. Sociocultural information, medical information, and data from a short clinical examination were collected anonymously into a standardized case report form at sampling.

3.2. Laboratory methods

3.2.1. Biological sampling

Three sponge brushes (Oragene®-DNA kit DNAGenotek™ Ottawa, Canada) inserted in the mouth for 30 s each were placed in Oragene®-DNA medium. The quantity of saliva was estimated by weighing.

3.2.2. Detection of CMV DNA

DNA was extracted from 400 μL of sample using BioRobot EZ1 Workstation (Qiagen, Hilden, Germany), eluted in 120 μL, and 10 μL were analyzed by real time PCR targeting *UL83* gene (cut-off 500 copies/mL) [19]. Albumin gene quantification, positive and negative controls in each CMV run, provided a validation of the whole process.

3.2.3. Genotyping.

Genotyping was performed on envelope glycoproteins gB (from both AD2 and CLZ encoding regions), gN and gH as described [20]. This method allows clonality analysis (one strain defined by identity of gB-AD2, gB-CLZ, gH and gN genotypes).

3.3. Statistical methods

Sample size: In the DCC group to assess the feasibility of the salivary sampling for a hypothesized 50% acceptability, a 10% precision for 95% confidence interval (95%CI) and a cluster effect of 2, a 195 children sample was necessary.

In the EU group to assess prevalence of CMV shedding for a 5% hypothesized prevalence and a 2% precision for a 95% confidence interval, a 460 children sample was necessary.

Statistical analysis: Analyses were performed using SAS v 9.1 software (SAS Institute, Cary, NC, USA). Quantitative variables were expressed as median and interquartile range (IQR). Qualitative variables were described using percent and 95%CI. Confidence intervals for percentages in DDC group integrated a cluster effect.

Univariate logistic regression analysis (using proc logistic) was used to assess association between positivity of PCR and sociodemographic or clinical characteristics of children. When considering such logistic regression in the DCC group, we integrated random effects for center effect using the GLIMMIX procedure, which fits statistical models to data with correlations or nonconstant variability and where the response is not necessarily normally distributed. These models are known as generalized linear mixed models (GLMM). The GLMMs, like linear mixed models, assume normal (Gaussian) random effects. Conditional on these random effects, data can have any distribution in the exponential family.

Quantitative variables were compared using Mann–Whitney test or Kruskall–Wallis test when appropriate, and Fisher's exact test. Correlation between percent treated as quantitative variable and the center size was assessed using Spearman's rank test. A p-value under 5% was considered as statistically significant.

4. Results

4.1. Analytical process validation

The average volume of saliva added in the medium was 433.73 μL (95%CI: ±17.69) and average DNA concentration was 30.46 ng/μL (95%CI: ±2.6), showing reproducible sampling and extraction, confirmed by albumin gene quantification. All the PCR runs were validated, without any contamination detection.

4.2. CMV shedding

4.2.1. DCCs

We included 256 children (51.6% males (109/211), 48.3% females (102/211), 45 missing values), median age 20.48 months (IQR, 12.13–27.97), recruited from six DCCs (from centers 1 to 6: 40, 26, 9, 79, 23 and 79, respectively); 37 were not included due to parental opposition (8, 1, 3, 0, 8 and 17, respectively). Overall acceptability in DCCs was 87.3% (95%CI 78.5–96.2). Parents socio-economic level was: medium-high income 48.44% (124/256), low income 47.66% (122/256), unemployed 3.9% (10/256). CMV shedding prevalence was 51.9% (95%CI 22.8–81.1), from 0.0% (0/9) to 74.7% (55/79) (respectively 22.5%, 7.7%, 0.0%, 74.7%, 34.8% and 69.6%) (Fig. 2). There was a positive correlation between the center admission capacity and CMV shedding (rho = 0.81, $p = 0.0499$) but no correlation was observed with parental socio-economic level. Viral load in saliva did not differ significantly between DCCs (Kruskall–Wallis test, $p = 0.90$), with the overall median CMV DNA level being 5.40 $\log_{10}$ copies/mL (IQR, 4.65–6.37) and a range from 5.32 $\log_{10}$ copies/mL in center 4 (59/79 CMV shedding toddlers) to 5.76 $\log_{10}$ copies/mL in center 5 (8/23 CMV shedding toddlers). The distribution of shedding was unimodal, with one peak at 5.5 $\log_{10}$ copies/mL (Fig. 3A). In the two DCCs with the highest prevalence of CMV shedding, there were one or two children with a very high level of CMV shedding (more than 9 $\log_{10}$ copies/mL). DCCs with lower prevalence of CMV shedding did not have children with high CMV viral loads in saliva. In univariate analysis presence of rhinorhea was associated with a 2.28 (95%CI 1.11–4.70, $p = 0.026$) increased risk of CMV excretion (Table 1). Two groups were compared: a high viral load group with more than 6 $\log_{10}$ copies/mL (87/133 children: 65.4%) and a low viral load group (46/133 children: 34.6%). The average age of children in the high level group was