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Viral shedding, and distribution of cytomegalovirus glycoprotein H (UL75), glycoprotein B (UL55), and glycoprotein N (UL73) genotypes in congenital cytomegalovirus infection

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ABSTRACT

Background: Children with congenital CMV infection (cCMV) shed virus in urine and saliva for prolonged periods of time. Outcome of cCMV varies from asymptomatic infection with no sequelae in most cases, to severe longterm morbidity. The factors associated with asymptomatic cCMV are not well defined. We evaluated the viral shedding in a cohort of infants with cCMV identified on newborn screening. In addition, we describe the distribution of viral genotypes in our cohort of asymptomatic infants and previous cohorts of cCMV children in the literature.

Methods: Study population consisted of 40 children with cCMV identified in screening of 19,868 infants, a prevalence of 2/1000. The viral shedding was evaluated at 3 and 18 months of age by real-time CMV-PCR of saliva and plasma, and CMV culture of urine. CMV positive saliva samples were analyzed for genotypes for CMV envelope glycoproteins gB (UL55), and gH (UL75) by genotype specific real-time PCR, and gN (UL73) by cloning and sequencing

Results: At 3 months age 40/40 saliva and urine samples, and 19/40 plasma samples were positive for CMV. At 18 months age all urine samples tested (33/33), 9/37 of saliva samples, and 2/34 plasma samples were positive for CMV. The genotype distribution did not differ from the published data

Conclusions: The urinary virus shedding is more persistent than salivary shedding in children with cCMV. The genotype distribution was similar to previous literature and does not explain the low disease burden of cCMV in our population.

1. Background

Approximately 0.6 % of children in the developed countries acquire congenital CMV infection (cCMV) during fetal life [1,2]. Most infants with cCMV and those who acquire CMV infection early in life continue to shed the virus in urine and saliva for prolonged periods of time [3]. Following primary CMV infection, virus shedding can be detected intermittently during periods of reactivations of the persistent infection or infection with new virus trains (reinfections) [3,4].

CMV has extensive genetic diversity. Envelope glycoproteins have an important role in host immune response and viral replication [5,6]. Variability in the genes encoding these proteins have been speculated to contribute in the virulence of the strain.6] Glycoprotein B, the major component of the lipid envelope, plays an important role in the virus entry and cell-to-cell spreading. Glycoprotein H and its complexes with other glycoproteins are involved in the fusion of the virus to the host cell membranes, essential step for the viral entry into the cells. Glycoprotein N is involved in the virus attachment to the host [5]. However,

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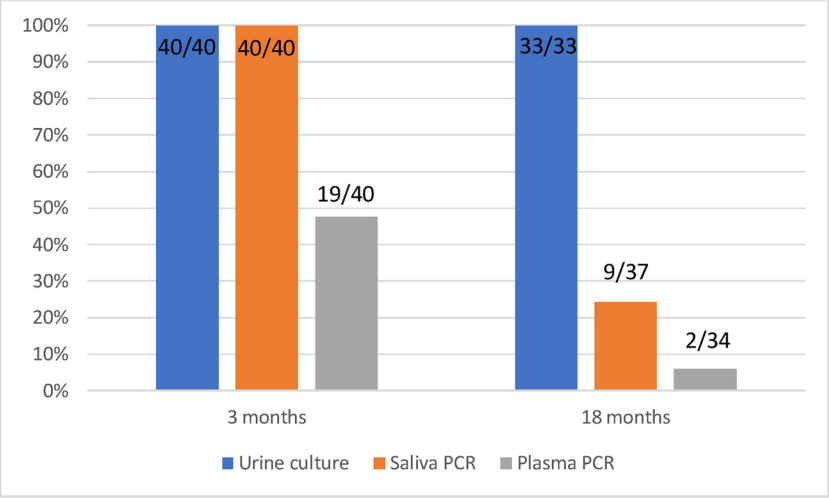

Fig. 1. Viral excretion in urine, saliva, and plasma at 3 months and 18 months age.

the current knowledge about the virulence factors among different strains is incomplete.

In Finland the disease burden of cCMV has been low. The prevalence of cCMV was only 2 in 1000 [7]. Additionally, in all University hospitals covering about 30 000 births per year, only 29 children with symptomatic cCMV infection had been identified between 2000 and 2012 [8].

2. Objectives

The objective of our study was to describe the viral shedding patterns during the first 18 months of life in Finnish children with cCMV, identified on newborn screening. In addition, the distribution of genomic variants of envelope glycoproteins of CMV, glycoprotein B (gB, *UL55*), glycoprotein H (gH, *UL75*) and glycoprotein N (gN, *UL73*) in saliva samples from infected infants was examined and compared to other cohorts in the literature. We also evaluated the association of genotypes and viral excretion in the outcome of cCMV in our cohort.

3. Study design

The study population consisted of 40 children with cCMV identified on screening of 19,868 infants born in Helsinki area Hospitals from September 2012 to January 2015. The findings from the screening study has been described earlier [7]. Informed consent was obtained from the parents for the enrollment in the screening. Newborn CMV screening was carried out by testing of saliva samples obtained during the first week of life using a real-time PCR assay as described [7,9]. The follow-up saliva, urine, and plasma samples were collected at 3 months and at 18 months age. CMV shedding in the follow-up saliva samples was determined using the same real-time CMV PCR assay [9]. The saliva CMV-PCR was considered positive if one or more genomic equivalents of CMV DNA per reaction were detected. The limit of detection for genotyping PCR assay is 200 ge/ml. The plasma samples were tested by a commercial assay (COBAS AmpliPrep/COBAS Taqman CMV test, Roche) according to the manufacturer´s instructions to determine viral load. The analytical sensitivity of the commercial assay is 56 IU/ml and linear range 137 IU/ml – 9.1 E 6 IU/ml. Urine samples were tested by a rapid culture method to detect the presence of early CMV nuclear antigen by immunofluorescence test, ≥1 cell showing a typical positive nuclear signal was considered a positive finding [10].

The serial saliva samples from children with cCMV collected during the first week of life, 3 months age, and 18 months age that were positive for CMV DNA were analyzed for genotypes of envelope glycoproteins, gB (*UL55*) and gH (*UL75*) using a genotype-specific real-time PCR as previously described [11–14]. The screening saliva samples were also examined to determine gN (*UL73*) genotypes by cloning of the amplified gN and screening colonies as previously described [15].

The neurologic outcome of the children at 18 months age was evaluated by the Griffiths Developmental Scales and the hearing was tested with transient evoked otoacoustic emission (tEOAE) and sound field audiometry (SF) as described earlier [7].

Statistical analyses were performed with IBM SPSS Statistics 22. Mann-Whitney *U* test was used to determine if the viral genotype, positive DNAemia at 3 months age, or persistent salivary excretion of CMV at 18 months of age were associated with hearing and neurodevelopmental outcomes and to compare the viral loads between symptomatic and asymptomatic children. The Fisher´s exact test was used to explore the association of different genotypes with the symptomatic and asymptomatic infections.

4. Results

Of the 40 infants with confirmed cCMV identified in a prospective newborn CMV screening, 4 had symptomatic infection and the remaining 36 infants were asymptomatic. The results of the newborn CMV screening and outcome have recently been reported [7]. One child was classified as symptomatic due to microcephaly, and three due to calcifications seen in the cerebral ultrasound. The outcome at 18 months age did not differ from that of the healthy controls [7].

4.1. Virus shedding

The saliva and urine samples collected at 3 months of age from all 40 infected infants were positive for CMV by real-time PCR and culture, respectively. At 3 months of age, plasma samples from 19/40 (48 %) were positive for CMV by PCR with a viral load between 56 and 753 IU/ml. Urine samples were collected from 33/40 children at 18 months of age and all of those were positive by culture. The saliva PCR was only positive in 9/37 (24 %) samples collected at the 18-month visit. CMV DNAemia was present in only 2/34 plasma samples collected at 18 months of age. Both positive plasma samples had CMV DNA level under the linear range 137 IU/ml. All three sample types (saliva, urine, and plasma) collected at both 3-month and 18-month visit from 32 children were tested. Both children with positive plasma CMV PCR at 18 months were positive for urine culture and the saliva sample from one infant was also positive. Fig. 1.

The viral load in the screening saliva sample at birth, or viral load in the saliva or plasma sample collected at the 3-month visit did not differ significantly between symptomatic and asymptomatic children. The

CMV DNAemia at 3 months age or persistent saliva CMV-PCR positivity at 18 months age was not associated with neurodevelopmental performance in the Griffiths Developmental Scales or hearing thresholds at 18 months age.

4.2. CMV envelope glycoprotein genotypes

Screening saliva samples (gH, gB, and gN): Of the screening saliva samples collected from the 40 infants with confirmed cCMV during the first week of life, 38 were available for genotyping. The gH (*UL75*) genotyping was completed for 34 samples. Both gH1 and gH2 were distributed equally, 18/34 for each genotype. Two screening samples contained both gH1 and gH2 genotypes (6 %). The glycoprotein B (gB, *UL55*) genotyping could be accomplished in 37 samples and all samples contained a single gB genotype. The gB1 was the most common genotype (19/37, 51 %) followed by gB3 (9/37, 24 %), gB2 (7/37, 19 %), and gB4 (2/37, 5%). Genotyping of glycoprotein N (gN, *UL73*) was completed in 24 screening saliva samples. The most common genotype was gN1 in 7/24 (29 %), followed by gN4c in 6/24 (25 %), gN3b in 5/24 (21 %), gN4a in 3/24 (13 %), and gN3a and gN4b in 2/24 (8%) samples. One sample had multiple gN genotypes, gN1 and gN4a (4%). New gN mutations resulting in amino acid changes that have not been previously described were observed in four specimens. A comparison of the distribution of the gH, gB, and gN in our cohort and in other cohorts reported in the literature is presented in Fig. 2 [14,16–37].

4.3. Analysis of follow-up saliva samples for gH and gB genotypes

Among the study children with genotyping results for gH and gB genes in screening saliva samples, the follow-up samples collected at 3 months of age were analyzed for gH (29/34) and gB (34/37) genotypes. The same gH and gB genotypes that were present in the screening sample were observed in the 3-month sample in all cases. The two samples that had both gH genotypes in the screening sample, only one gH genotype (gH2) was detected in the three-month saliva sample.

Only 9/37 saliva samples collected at 18 months of age were positive for CMV and of those, genotyping could be completed for 5. In four of the 5 samples, a different gH or gB genotype was present in the 18-month sample compared to screening and 3-month samples. The five children with genotyping results from all time points, screening, 3 month and 18 months, are presented in Table 1. The genotype remained same in the screening and 3 months but changes in most cases by 18 months age.

The genotypes of envelope glycoproteins, gH, gB, and gN were associated with neither symptomatic infection nor neurodevelopmental outcome as measured using Griffiths Mental Development Scales General Quotient.

5. Discussion

We have characterized virus shedding, and the distribution of genotypes of envelope glycoproteins gB, gH and gN in children with congenital CMV infection identified in a large prospective newborn CMV screening study. At 18 months age all children had persistent virus shedding in urine in contrast to only 24 % of saliva samples. The genotype distribution in the screening saliva samples was similar to the reports from other cohorts in the literature. Children with cCMV shed virus for prolonged periods of time [3,38]. Consistent with previous studies, we also observed that urinary virus shedding was more common (33/33) than salivary shedding (9/37) at 18 months age [3,4,39].

In our cohort of predominantly asymptomatic infants, about half of the infected infants (19/40, 48 %) were viremic at 3 months of age. The finding is similar to that reported by Forner et al. who observed CMV DNAemia at 3 months age in 50 % of 33 children with asymptomatic cCMV born following maternal primary infection during pregnancy [40]. Ten of 33 children (30 %) in that study developed long-term sequelae such as SNHL, hemiparesis, or intellectual impairment [40]. This is in contrast to findings in our cohort demonstrating favorable outcome at 18 months age [7]. The significance of DNAemia at 3 months of age with respect to long-term outcome is not known. Some studies have reported that higher viral loads are associated with long-term sequelae [3,41] but other studies did not find this association [42]. In our cohort, viremia at 3 months age was not associated with lower performance in Griffiths Mental development testing at 18 months age. The viral loads in plasma measured at 3 months age were low in all positive cases ($\leq$753 IU/ml).

Young children shedding the virus after congenital or postnatal infection are considered the most important source of infection to pregnant women [43]. Our finding that less than a third of children with cCMV continue to shed virus in saliva at 18 months is reassuring and this rate of salivary virus shedding is similar to the reported rates of shedding in healthy children attending day care [44,45]. Our findings suggest that children with cCMV infection should be treated as other children using standard hygiene precautions.

The distribution of genotypes of CMV glycoproteins, gH, gB and gN in cohorts of infants with cCMV reported in literature and observed in our study was shown in Fig. 2. The gH genotype distribution appears to be similar in different geographic locations and populations [14,16–18,36]. Both gH1 and gH2 genotypes seems to be equally distributed in the cohorts from USA, Poland, Austria, the Netherlands, and in Pakistan.16–18,36] The genotype distribution was similar in different populations regardless whether the cohort consisted mainly of asymptomatic (Finland and USA) or symptomatic infants (Poland) [14,16].

However, the distribution of gB and gN genotypes showed more variation between populations [14,17–27,29,31,32,34–37]. In our cohort, similar to most other studies, all four gB genotypes were present with the dominance of gB1 in half of the samples [14,17–23,28,36]. In a Brazilian cohort of 47 asymptomatic and 2 symptomatic infants, only genotypes gB1-3 were present [33]. In cohorts from Poland, the genotype gB4 was missing in a cohort of 60 mainly symptomatic (72 %) children and gB3 and gB4 were absent in a small cohort of 12 children [19,21]. A Japanese cohort with 31 mostly asymptomatic children, had only gB1 and gB3 genotypes. 23] In Mexico, samples from 5 children with cCMV included only gB1 and gB2. 28] The CHIMES study in the USA was the only study to report gB5 genotype [14].

The distribution of gN genotypes in infants with cCMV was described in 7 published reports [14,18,29,31,32,34,37]. All 7 g N genotypes were present with even distribution, except gN2 was absent in our cohort, the Pakistani, and Brazilian cohort.18,29] The small cohort from Brazil with 14 children lacked also gN3a and 3b genotypes.29] In the Italian cohort from 2003, only four gN genotypes gN1, gN2, gN3, and gN4 were reported. 34] However, gN genotyping data from our study should be interpreted with caution because genotyping was only completed for 24/40 infants.

Mixed infections were infrequent in our cohort. However, even studies that observed higher rate of mixed infections did not find a significant association between mixed infections and symptomatic infection or sequelae [14]. The children in our cohort were mostly asymptomatic and the outcome at 18 months was favourable [7]. The number of infected infants and few children with mixed infection does not allow us to make meaningful conclusions on the role of mixed infections and outcome.

We observed same genotypes in samples collected at birth and at 3 months of age. However, in the small number of children in whom genotyping was carried out in samples obtained at all three time points, the 18-month sample contained a different genotype from the one seen at birth and 3 months of age. It is possible for these children may have been reinfected with a different strain from exposures in day care. However, the small number of infants with data at all three time points limits the utility of this finding.

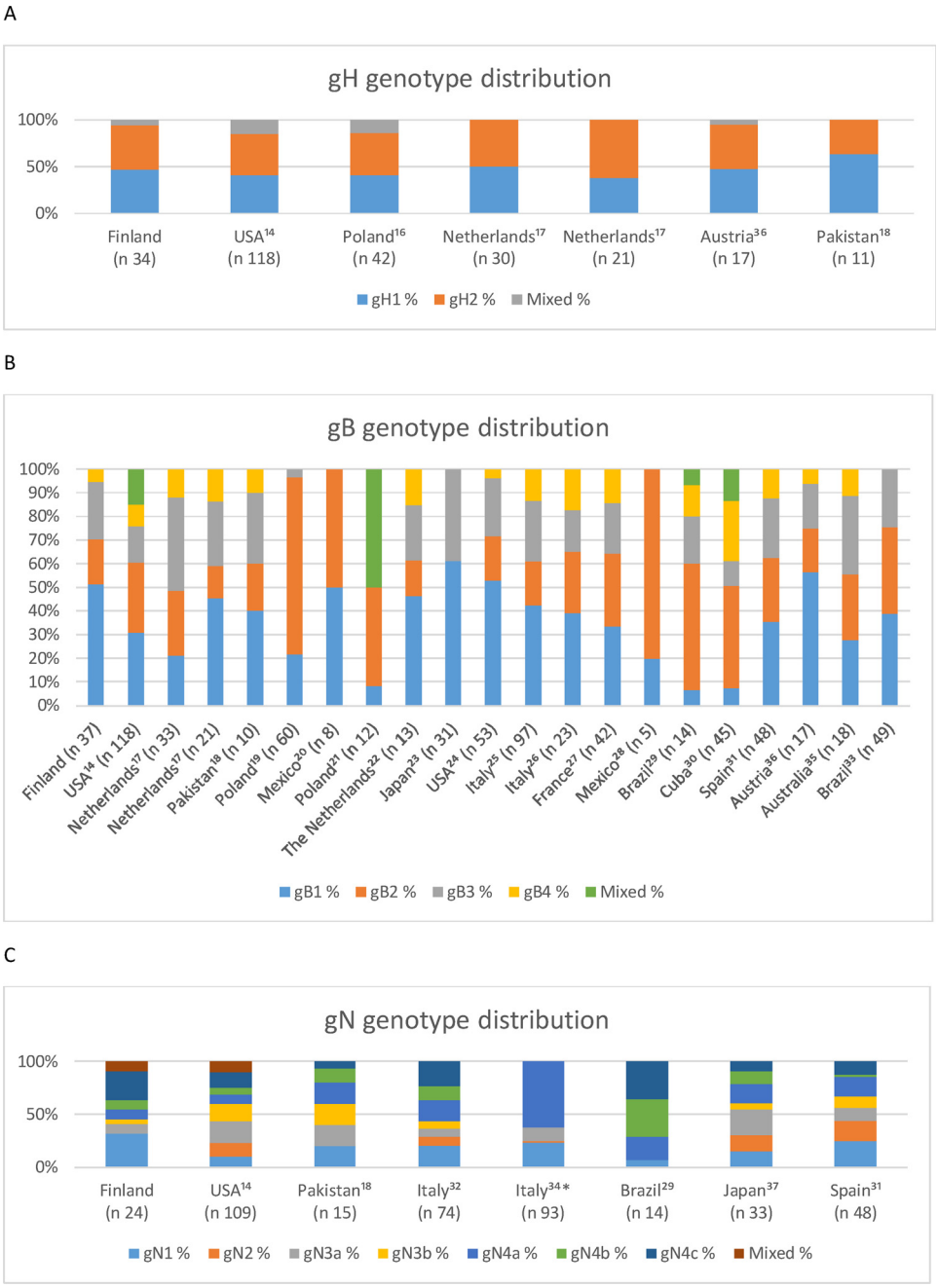

Fig. 2. Distribution of the genotypes of envelope glycoproteins, gH (A), gB (B) and gN (C) in our cohort from Finland and in other cCMV populations in the literature. *only gN4 reported instead of gN4a, gN4b, gN4c

Table 1
The gH and gB genotypes of the cases with genotyped CMV positive saliva samples for all time points: screening, 3 months and 18 months.

case	Screening saliva	3 month saliva	18 month saliva
1	gH2, gB3	gH2, gB3	gH1, gB4
2	gH1, gB2	gH1, gB2	gH1, gB no amp
3	gH2, gB2	gH2, gB2	gH1, gB no amp
4	gH2, gB1	gH2, gB1	gH no amp, gB4
5	gH1, gB2	gH1, gB2	gH1, gB3

no amp = could not be amplified.

The strengths of our study are that we report the virus shedding and genotyping data from an unselected cohort of children with cCMV identified on screening. Most studies in the literature reporting genotyping data present either children with symptomatic infection or a smaller convenience sample of a larger cohort. A limitation of the study is the small number of symptomatic children, only 4 children. It is impossible to draw conclusions about the differences in genotypes or viral shedding between symptomatic or asymptomatic children. Another limitation is that the genotyping could not be performed for all saliva samples.

In conclusion, the genotype distribution in our unselected population-based cohort did not differ from the previously described cohorts. Thus, the genotype distribution is unlikely to explain the relatively low disease burden of cCMV reported in Finland [7,8]. Although all congenitally infected infants were shedding virus in the urine still at 18 months age, less than third of infected children were shedding the virus in saliva.

Ethical approval

The study protocol was approved by the ethics committee for women, children, and psychiatry in the Hospital District of Helsinki and Uusimaa

Transparency document

The Transparency document associated with this article can be found in the online version.

CRediT authorship contribution statement

Laura Puhakka: Conceptualization, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **Sunil Pati:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing. **Maija Lappalainen:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing - review & editing, Supervision. **Tuula Lönnqvist:** Conceptualization, Writing - review & editing, Supervision. **Riina Niemensivu:** Conceptualization, Writing - review & editing, Supervision. **Päivi Lindahl:** Conceptualization, Writing - review & editing, Supervision. **Tea Nieminen:** Conceptualization, Writing - review & editing, Supervision. **Raija Seuri:** Conceptualization, Writing - review & editing. **Irmeli Nupponen:** Conceptualization, Writing - review & editing. **Suresh Boppana:** Conceptualization, Methodology, Validation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision. **Harri Saxen:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

S. Boppana has been consultant in CMV Vaccine Advisory Committee, Merck, Inc. Other authors declare no conflicts of interest.

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