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Short communication

Analysis of human cytomegalovirus strain populations in urine samples of newborns by ultra deep sequencing

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ABSTRACT

Background: Different human cytomegalovirus (HCMV) strains may persistently coexist in the human host. In immunosuppressed patients infection with mixed HCMV populations was associated with a more severe course of infection. Congenital HCMV infection may lead to severe fetal disease and possibly mixed HCMV strain infections might have also impact on the clinical consequences for the newborn. Mixed HCMV strain populations were so far detected in saliva but only rarely in urine of congenitally infected newborns.
Objectives: We have therefore analyzed the extent of mixed HCMV genotype populations in urine of congenitally infected newborns using a highly sensitive deep sequencing method.
Study design: Twenty urine samples (17 initial and 3 follow-up samples) from 17 congenitally infected newborns with a median HCMV DNA load of 7.5 $\log_{10}$ copies/ml were included. Deep sequencing was applied for gO (UL74) genotyping and quantitative real-time PCR assays were used for gB (UL55) and gH (UL75) genotyping.
Results: In none of the urine samples a gO genotype mixture was detected, although a mean of 10.000 sequence reads per amplicon was analyzed, which allows to explore gO genotypes down to less than 1% of the total gO sequences. Also only one gB genotype was detected in the patients' initial samples, while a gH genotype mixture was detected in one case using real time PCR with a sensitivity of 5% for minor populations.
Conclusion: Mixed HCMV genotype populations are only rarely found in urine of congenitally infected newborns even when highly sensitive HCMV genotyping methods are applied.

1. Background

Human cytomegalovirus (HCMV) infection during pregnancy may lead to congenital infection of the fetus and severe clinical complications in the child [1,2]. HCMV transmission from mother to fetus seems to occur in 30–40% of primary infections, but also HCMV reinfection in presence of pre-existing maternal immunity to HCMV may lead to intrauterine transmission [3,4].Diagnosis of congenital HCMV infection is made by detection of virus in urine, saliva, or blood within the first 2–3 weeks of birth [1].

HCMV establishes a persistent infection over life time, and may reactivate [1]. In addition, reinfections with different HCMV strains may occur [5,6] and multiple HCMV strains may persistently coexist [7]. In transplant patients infections with mixed HCMV strains appear to be associated with a more severe course of infection and disease [8–13]. Thus, also the question to which degree mothers may transmit more than one HCMV strain to the fetus could be clinically relevant. From previous investigations it was suggested that mixed HCMV populations may be frequently detected in saliva of congenitally infected newborns [14,15], while multiple HCMV strains were rarely found in urine of newborns using genotype-specific real-time PCR and Sanger sequencing methods [15–18]. It is, however, unclear whether virus strain mixtures were

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Table 1
Distribution of HCMV genotypes in urine of 17 congenitally infected newborns.

Subject	Age (in days)	HCMV DNA load (log_{10} copies/mL)	Genotype gO[a]	gH[b]	gB[b]
P3	8	6,7	1a	1	1
P12	2	7,5	1a	1	1
P16	16	8,0	1a	1	2
P4	2	7,9	1b	1	1
P5	13	7,6	1b	1	3
P6	12	7,3	1b	1	1
P11	8	5,6	1b	1	1
P15	2	9,9	2a	1	4
	98	5,6	2a	1	4[c], 1
P1	5	7,8	2a	2	1
P7	8	7,2	2a	2	1
P9	3	8,7	2b	2	1
P2	2	9,1	3	2	3
P10	8	7,0	3	2	1
	32	7,8	3	2	1
	90	8,5	3	2	1
P13	13	7,8	3	2	1
P18	18	7,4	3	2[c], 1	2
P8	1	4,6	5	2	2
P14	9	6,3	5	2	3

[a] Quantitative gO genotyping by ultra-deep pyrosequencing [19]; the sensitivity limit for minor genotypes was 0.1% for patient samples, P6, P9, P10, P11, P14, P15, P18, and 1% for all other samples due to the number of sequence reads per sample.
[b] gB- and gH genotyping by quantitative real-time PCR assays [21].
[c] Major HCMV genotype (>80% abundance).

undetectable in urine due to the limited sensitivity of previously used methods for minor populations.

2. Objectives

We therefore analyzed whether mixed HCMV genotype infections can be found in urine samples of congenitally infected newborns by ultra deep pyrosequencing (UDPS).

3. Study design

In the present study all newborns were selected, of whom urine samples were sent to our department between 2001 and 2011 due to suspected congenital HCMV infection and who were diagnosed as congenitally infected by HCMV DNA detection in urine within 18 days of birth. From 17HCMV positive newborns, a total of 20 frozen (−20 °C) stored urine samples were still available, consisting of 17 initial (day 2-day 18 after birth) and 3 follow-up samples (days 32, 90, and 98). The virus loads in original samples significantly correlated with that in the thawed samples when included in the study (Table 1) as identified by Cobas Amplicor CMV Monitor Test (Roche Molecular Systems, Germany). The study was approved by the local ethics committee (EK-number: 1679/2012).

HCMV DNA was isolated using the automated NucliSENS® easyMAG® system (BioMérieux, France). UDPS gO genotyping was applied to all samples as previously described [19]. Briefly, the HCMV gO gene region (AD169 position 106555–106853) was amplified using primers composed of 454 adaptors, multiple identifiers and gO-specific sequences. Purified PCR products were quantified, pooled and subjected to UDPS using the GS FLX Titanium sequencing kit in a 454/Roche GS FLX instrument. A mean of 10.611 sequence reads, ranging from 1.998 to 25.154 per sample was achieved after passing the default GS FLX quality filters and after pre-processing using RDP's pyrosequencing pipeline (http://pyro.cme.msu.edu/). All sequence reads per sample were searched for individual gO genotype signature sequence patterns using self-developed Perl script with regular expressions and all reads that did not match with a well-known gO genotype pattern were aligned (ClustalX 1.83), and manually edited (BioEdit 7.0.1). Unique sequence reads were combined into a single gO alignment, from which a neighbor-joining tree was inferred using the Kimura 2-parameter method in MEGA 4.0 [20]. Further, gB- and gH-genotyping was performed by previously described genotype specific real time PCR assays [21]. To investigate potential PCR interferences, HCMV DNA negative urine of neonates spiked with HCMV strains Merlin and AD169 (ratio: 5–5%; total load: 10^7 and 10^6 copies/ml) showed accurate quantitation of both genotypes as tested by genotype-specific real-time PCR. The detection limit for minor genotypes in mixtures was 5% for the real-time assays [21] and 0.1–1% for UDPS depending on the number of sequence reads as previously described [19].

4. Results

The overall results obtained by genotype analyses are summarized in Table 1. In all 20 urine samples only one genotype was detected at the gO gene. All gO genotypes except gO1c and gO4 were represented in the subject population, with gO1b and gO3 being the most common genotypes (23.5% each). All individual gO genotype sequences showed 100% identity with reference sequences (Fig. 1). There was no evidence of minor gO genotypes or variants thereof at a frequency of down to 0.1% that coexisted along with the major gO genotype.

Next, genotype-specific PCR analyses were performed on the gB and gH gene to further expand the HCMV strain characterization. In the initial urine samples always only one gB genotype was detected. As shown in Table 1, all four major gB genotypes were represented in the patients, with gB1 being the predominant genotype (59%). Also, both gH genotypes were represented and in one case a mixture of both gH genotypes was found at a concentration of 95% for gH2 and 5% for gH1.

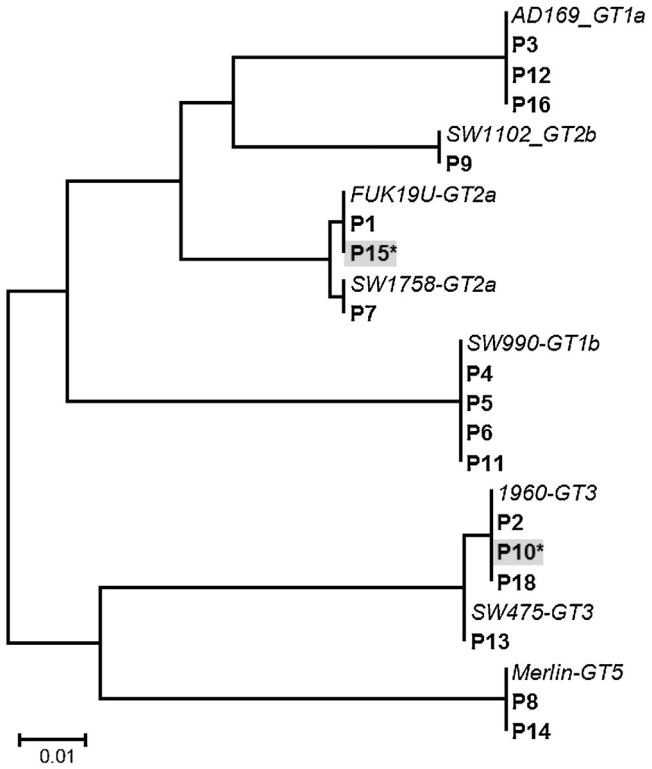

Fig. 1. Phylogenetic analysis of all unique gO genotype sequences. Neighbor-joining tree was constructed using the unique gO genotype sequences found in the urine samples of congenitally infected newborns. The tree illustrates the clustering with known prototype genotype sequences (shown in italics).