CMV DETAIL (Improving iDEntification of maTernAl CMV reInfection and characterization of congenitaL CMV strains)

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SUMMARY

Title	CMV DETAIL (Improving iDEntification of maTernAl CMV reInfection and characterization of congenitaL CMV strains)
Goal	To characterize maternal CMV (re)infection and infant congenital infection using serologic and molecular analyses that interrogate the entire viral genome.
Objectives	1. Determine the proportion of maternal CMV primary versus non-primary infection among mothers of cCMV infants using standard serologic methods and compare the maternal and newborn clinical presentation between primary and non-primary cases. 2. Determine the proportion of mothers with and without cCMV-affected infants that have evidence of reinfection using innovative serologic methods. 3. Characterize the viral strains resulting in primary and non-primary cCMV infection.
Timeline	August 2022 – July 2024
Project design	This study will employ a retrospective cohort design with a prospective component to maximize sample size.
Sample collection and time points	Samples collected from pregnant women in BC and their infants as part of routine care from 2018-2024. Samples include maternal blood and infant samples such as serum, saliva, or urine.

1.0 BACKGROUND

1.1 Epidemiology of Cytomegalovirus (CMV)

As the most common congenital infection and leading cause of non-genetic childhood hearing loss and neurocognitive disability, CMV causes an enormous disease burden globally (1,2). CMV has a ~235 kb double-stranded DNA genome, the largest of any human herpesvirus. Despite being a DNA virus, CMV's genome is highly genetically diverse, both within and between individuals (3,4) and infection with multiple distinct strains is well-described (5–8). Many people become infected in early childhood (9), however, natural immunity is incompletely protective, evidenced by the fact that congenital CMV infection (cCMV) can occur among women infected prior to conception (10,11). Maternal CMV replication, which drives transmission to the fetus or infant, can occur from incident infection, reinfection or reactivation of latent infection during pregnancy.

1.2 Study Rationale

Increasing evidence suggests that, worldwide, maternal reinfection with a new strain of CMV during pregnancy may be the main cause of cCMV, as opposed to primary infection or reactivation during pregnancy (5,10–12). However, the rate of maternal reinfection has not been well quantified. The rate of maternal reinfection with non-primary cCMV has been estimated to be between 13-28% (13,14). These estimates were based on a strain-specific enzyme-linked immunosorbent serologic assay (ELISA) that only discriminates between a small number of CMV strains (5,14,15), so the true incidence may be much higher (13,15,16). Indeed, mathematical modeling by our group suggests that >50% of non-primary cCMV is due to maternal reinfection (17).

A CMV vaccine is a high global health priority. So far, CMV vaccines that have been tested in clinical trials have been strongly protective against primary infection, but there is no evidence of their efficacy to reduce reinfection or non-primary cCMV. To guide the development of an effective vaccine that prevents both non-primary and primary cCMV, a better understanding of maternal reinfection and of the viruses transmitted congenitally, are required. We are currently applying our CMV-Scan assay to accurately define the rate of reinfection during pregnancy in large prospective cohorts; however, given the relative infrequency of cCMV (<1%), these cohorts do not allow us to look at associations with cCMV infection. As such, this study, which takes advantage of samples accrued by a provincial laboratory on a population-wide scale, represents a natural next step in our research.

2.0 OBJECTIVES

1. Determine the proportion of primary maternal CMV infection versus non-primary infection among mothers of cCMV affected infants using standard serologic methods and compare the maternal and newborn clinical presentation between primary and non-primary affected infants.
2. Determine the proportion of mothers with and without cCMV-affected infants that have evidence of reinfection using innovative serological methods.*
3. Characterize the viral strains resulting in primary and non-primary cCMV infection.

* The number of reinfections will be compared between mothers of cCMV-affected infants from the CMV-DETAIL cohort and seropositive mothers with cCMV-unaffected infants from our ongoing, separately funded, Canadian cohort study. Any CMV-DETAIL prospective cases with a presumed diagnosis of CMV infection have an infant that is cCMV-unaffected will also be used for this objective.

3.0 STUDY DESIGN

This study will employ a retrospective cohort design (Section 3.1) to ascertain the nature of maternal infection associated with infant cCMV infection.

A prospective component (Section 3.2) will permit understanding of the nature of maternal infection and the evolution of infection for both cCMV infected and uninfected infants allowing identification of co-factors associated with infant infection.

3.1 Retrospective Study Design (2018 – 1 Jul 2023)

Clinical maternal blood samples are routinely collected in the first trimester of pregnancy and near delivery in BC to screen for congenitally infectious agents, and stored for ≥1 year at the BC Centre for Disease Control (BCCDC). Serum, saliva, urine, products of conception, and anatomical pathology tissue samples from cCMV positive infants are also collected at birth and stored at the BC Children's Hospital Laboratory. Each year, ~9 infants with cCMV are clinically identified. We will leverage the stored samples from the past 4.5 years (2018 – **1 Jul 2023**) for the retrospective component of the study.

3.1.A Retrospective Inclusion Criteria

1. Have delivered an infant with a diagnosis of congenital CMV infection in British Columbia between January 2018 and **1 Jul 2023**.

3.1.B Retrospective Recruitment

Participants (mother-infant pairs) with a cCMV diagnosis from 2018 - **1 Jul 2023** will be clinically identified by Dr. David Goldfarb, Dr. Laura Sauvé and Dr. Deborah Money.

In his role as medical microbiologist, Dr. Goldfarb is aware of all cases of cCMV in BC as diagnostic testing for the province is performed at BC Children's Hospital (BCCH). As co-investigator on this project, Dr. Goldfarb will identify and provide access to samples for infants congenitally infected with CMV through the BCCH Laboratory, which is the provincial reference laboratory for cCMV urine and saliva testing. Dr. Sauvé (co-investigator) and Dr. Money (primary investigator) are also able to clinically identify cCMV cases through their roles as infectious diseases specialist physicians at the Oak Tree Clinic at BCWH, and through BCCH consultations.

For identified retrospective cases (2018 - **1 Jul 2023**), study staff will send the potential participants a letter of initial contact. If potential participants respond to the letter and would like their samples

included in the study, Dr. McClymont, or designate research staff, will complete the consent via phone or Zoom with emailed consent form using the approved phone script.

For those who do not respond to the letter, samples be included using a waiver of consent. A waiver of consent will also be applied to those cases that were a pregnancy loss (including stillbirth, therapeutic abortion, or spontaneous abortion).

3.2 Prospective Study Design (1 Jul 2023 – 2024)

As prospective cases are clinically identified in BC, we will leverage their clinical samples collected as part of the workup of maternal suspected CMV infection. Multiple maternal samples, cord blood samples, placental pathologic samples and infant samples are available for clinical investigations and subsequently stored at the BC Children's Hospital Laboratory or at the BCCDC. Prospective cases will include participants who delivered between **1 Jul 2023** to 2024.

3.2.A Prospective Inclusion Criteria

1. Pregnant women with a confirmed or presumed diagnosis of CMV infection in British Columbia between **1 Jul 2023** and December 2024

3.2.B Prospective Recruitment

Prospective cases (**1 Jul 2023** - 2024) will be asked by their care provider (the obstetrical and pediatric infectious diseases physicians consulting on congenital infections in pregnancy, which includes Dr. Sauvé and Dr. Money) during a clinical visit if they are comfortable being contacted by research staff to learn about the CMV DETAIL study. Dr. McClymont, or designate research staff, will then complete the consent process in person or via phone with emailed consent form using the approved phone script.

If any cCMV cases are identified in the BCCH Laboratory that were not seen by the BC Women's Hospital infectious disease physicians, a study staff member will reach out to the referring care provider to obtain the case's consent to be contacted by research to learn about the CMV DETAIL study. The participant would then be consented as described.

If a participant would not like to include their infant in CMV DETAIL but would still like to participate themselves, we are able to utilize their samples to assess the maternal rate of re-infection. If at any time participants reach out and wish to withdraw their consent, this will be respected and they will be withdrawn from the study.

If any enrolled prospective cases with a presumed diagnosis of CMV infection have an infant that is cCMV-unaffected, their samples will still be included in the study. These samples will be used as part of objective 2, which is to determine the proportion of mothers with CMV reinfection among those with and without cCMV-affected infants.

4.0 Laboratory Methods

All laboratory testing, including CMV ELISA and CMV-Scan assays, will be performed on maternal early pregnancy and delivery blood samples from both the retrospective and prospective

components of CMV DETAIL when available. Next generation sequencing (NGS) and CMV genomic analyses will be performed on infant samples collected from both the retrospective and prospective components of CMV DETAIL using methods that have been extensively validated by our group (10,18,19). All of these research laboratory tests will be performed in the Gantt/Boucoiran Lab in Montreal, QC

CMV IgG/IgM ELISA and IgG avidity testing:

CMV IgM and IgG seropositivity will be assessed with a commercial CMV ELISA (Platelia, BioRad). CMV IgG avidity will be performed when IgG and IgM are both positive and/or there is IgG seroconversion (negative to positive) during pregnancy to determine timing of infection; avidity is low early in infection and becomes high at 5-6 months post-primary infection (20,21). Although this method has been widely used to estimate primary versus non- primary CMV infection during pregnancy and the associated cCMV risk, it is unable to distinguish reactivation from reinfection. Thus, we will utilize this established and clinically used method alongside the novel CMV-Scan assay described below.

CMV-Scan:

CMV-Scan (22,23) allows comprehensive serological profiling using a phage display library that we have adapted to identify secreted antibodies to all (~400,000) variant epitopes among sequenced CMV strains, as defined by having <98% identity. The CMV DETAIL study team designed a library expressing all linear CMV epitopes, as 56 amino acid peptides overlapping by 46 amino acids, using open reading frames from GenBank plus our unpublished CMV NGS data from other cohorts. After removing redundant sequences, the corresponding oligonucleotide pools are cloned into T7 phage, and the library is expanded and validated by Illumina Sequencing (22,23). Briefly, we will incubate the CMV library with serum, immunoprecipitate the phages that bind to IgM or IgG with Dynabeads, and sequence the epitopes displayed by the captured phages on the Illumina platform, as described below (22,23). Controls will include CMV-uninfected serum, CMV-infected serum, library without serum, and no library/bead- only conditions. We previously implemented the original pan-viral VirScan library and more recently validated the coronavirus specific VirScan-CoV library (23); our existing VirScan bioinformatic pipeline has been adapted for analysis of data generated by the CMV-specific assay (24).

Next-generation sequencing:

The entire CMV genome will be sequenced from all available PCR-positive samples from women and newborns with cCMV using methods developed by our collaborators, and established in the Gantt laboratory (4,10,18,19). Extracted DNA is sheared by acoustic sonication (Covaris e220). DNA fragments will undergo end-repair, A'-tailing, and (Illumina) adaptor ligation. DNA libraries are then hybridized with biotinylated 120-mer custom RNA baits designed using all available CMV full genomes in GenBank for 16–24 hr at 65°C and subsequently bound to MyOne Streptavidin T1 Dynabeads (ThermoFisher Scientific). Following washing, libraries are amplified (18 cycles) to generate sufficient input material for Illumina sequencing. Paired-end deep-sequencing will be performed on an Illumina MiSeq using the 500 cycle v2 Reagent Kit (Illumina, MS-102–2003). The resulting millions of 2×250 bp paired-end reads will be trimmed and mapped by local assembly against a database of all fully or partially sequenced human CMV genomes available in GenBank. This approach reliably obtained high quality whole genome sequences from most clinical specimens with >3 log10 copies of CMV DNA/specimen from prior cohorts (4,10,19).

5.0 Statistical Analysis

We anticipate 30 cCMV cases with paired maternal and infant samples available. Prior data from CMV strain-specific serotesting suggests that the rate of reinfection is between 10-30% in pregnant women who transmit to their fetus (non-primary cCMV) (8,14). With the use of CMV-Scan, which is able to detect antibodies to all sequenced strains of CMV, we anticipate detecting a higher rate of reinfection. Our mathematical modeling predicts that over half of non-primary cCMV is due to maternal reinfection (17). Thus, we hypothesize that the reinfection rate detected will be >50% representing >8 reinfections. These high-dimensional data obtained with the proposed NGS and comprehensive serologic methods are well-powered for generating significant results from small sample sizes (19,24).

Objective 1: Determine the proportion of maternal CMV primary versus non-primary infection among mothers of cCMV cases using standard serologic methods and compare the maternal and newborn clinical presentation between primary and non-primary cases. Standard commercial CMV ELISA and CMV IgG/IgM and avidity testing will be used to define primary and non-primary infection, per current national guidelines (25). We expect that in this setting of ~50% adult seroprevalence, primary infection will account for ~50% of cases while non-primary infection will also account for ~50% (26,27). For infants seen at BC Children's Hospital, the provincial referral centre for pediatric infectious disease, the indication for CMV testing (symptomatic maternal infection, pregnancy complications, newborn symptoms, etc.) and newborn clinical presentation will be obtained from medical records, and compared between primary and non-primary cases.

Objective 2: Determine the proportion of mothers with and without cCMV-affected infants that have evidence of reinfection using innovative serologic methods. For CMV-Scan, the mean scores between first and last samples will be calculated using the BLOSUM62 substitution matrix, with a gap opening and extending penalty of −10 and −1, respectively (22,23). The maximum similarity score will be calculated between first and last samples for the center of the sliding window of each overlapping epitope. For each position in the 56-mer, the relative enrichment is calculated as the mean fold-change at that location relative to the median fold-change overall. In parallel, we will use emerging alignment-free representation of sequences allowing capture of non-linear relationships in sequence that cannot be held in an alignment (28). Thus, distance will be captured from strains with and without alignment.

Subsequently, we will use an incremental unsupervised machine learning approach to cluster samples into unique distinctive epitopes. The number of reinfections detected by CMV-Scan will be compared between mothers of cCMV-affected infants from the present cohort and seropositive mothers with cCMV-unaffected infants from our ongoing, separately funded, Canadian cohort study.

Objective 3: Characterize the viral strains resulting in primary and non-primary cCMV infection. NGS data on newborn specimens will allow us to characterize the CMV variants successfully transmitted congenitally. We will characterize the overall viral diversity of CMV populations using standard tools for de novo sequence assembly. Bayesian mixture models (HaROLD) will allow us to identify mixed infections, by ascertaining the most probable number of genotypes that describe the observed diversity (29). CMV genomic diversity is low early after primary infection and increases over time (3,30). To reconstruct the putative ancestral virus, we will use RAxML and PAML (31,32).

Sequences of the successfully transmitted CMV variants will be analyzed for motifs associated with congenital transmission in other populations (10). NGS results will be compared to CMV-Scan results generated for each mother to determine whether detection of a specific variant corresponds to a maternal pre-existing infection vs. reinfection.

6.0 Data Management / Stewardship

Data management will be performed by the coordinating centre, Reproductive Infectious Diseases Team, Women's Health Research Institute, a University of British Columbia Faculty of Medicine Centre at BC Women's Hospital and Health Centre in Vancouver, BC. Collected data will be entered into a REDCap database at the BC Children's Hospital Research institute. Each case will be anonymized and assigned a unique identification number (ID#). No direct personal identifiers will be included in the database.

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