CONGENITAL CYTOMEGALOVIRUS INFECTION: DISEASE BURDEN and SCREENING TOOLS

-Towards newborn screening-

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-Towards newborn screening-
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-Towards newborn screening-

Proefschrift

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Co-promotor

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*'I saw an **owl** up in a tree,*
I looked at him, he looked at me.
*How could I tell you of his **size**,*
for all I saw were two big eyes?'

Edna Hamilton
(contemporary Canadian poet)

Contents

Chapter 1

General introduction

General introduction

Human cytomegalovirus (CMV) was first isolated in cell culture in 1950 from the salivary gland and kidney of two infants who had died with enlarged or cytomegalic inclusion-bearing cells.[1] About 70 years before the identification of the causative agent, these cellular changes with a typical ***owl's eye*** appearance observed in affected newborns had led to use of the term cytomegalic inclusion disease (CID)[2]. After initially being called "salivary gland virus", the term "cytomegalovirus" was proposed by Weller et al in 1962.[3]

CMV

Human CMV or human herpesvirus (HHV) 5 is a member of the family *Herpesviridae*, which also includes the human viruses herpes simplex virus 1 and 2, varicella-zoster virus, Epstein-Barr virus, and HHV 6, 7 and 8. CMV has been sub-classified as a betaherpesvirus, originally based on its growth characteristics in vitro, but nowadays based on genetic sequence homologies.[4] Other beta-herpesviruses include HHV 6 and 7, the agents associated with the childhood disease exanthema subitum (sudden rash) or roseola infantum (rose rash of infants). CMV is amongst the largest human viral pathogens, measuring about 200 nm in diameter. The CMV virion consists of a capsid containing a large linear double stranded DNA genome encoding more than 200 potential proteins, enclosed by a protein tegument and a lipid envelope.[5] The tegument contains key regulatory proteins, among which the immunomodulatory protein pp65 (UL83).[4] This pp65 is the most abundant virion protein, accounting for about 15% of the total virion protein[4], and is the antigen that is detected in the diagnostic antigenemia assay. The capsid exhibits icosahedral symmetry of triangulation number (T) 16, with 162 capsomere subunits.[6] The envelope contains several glycoproteins with the most abundant ones being the gM (UL100)/gN (UL73) complex, gB (UL55), and the gH (UL75)/gL (UL115) /gO (UL74) complex.[7] The envelope renders the virus susceptible to lipid solvents, low pH, heat, and ultraviolet light. Unlike herpes simplex and varicella-zoster viruses, CMV exhibits an exceptionally broad cellular tropism, rendering CMV capable of infecting most cell types including dendritic, endothelial, epithelial, fibroblast, and monocytes/ macrophages.[5] A large number of cellular receptors for CMV have been proposed, mainly interacting with the CMV envelope glycoproteins gB, gH and gM.[5] The association of specific CMV glycoprotein genotypes with severity of disease has been addressed, with contradictory results.[8,9,10,11] There is little genetic homology between human CMV

and CMV of other species, restricting cell entry to human host cells and rendering humans the only reservoir. Primary infection results in lifelong latency with intermittent reactivation and excretion, applying the dictum 'once infected, always infected'. Viral latency is established within myeloid cells including myeloid progenitors, monocytes, macrophages, and dendritic cells. About 1 per 10,000 peripheral blood mononuclear cells of healthy seropositive individuals harbor several copies of the CMV genome[12], which is present in an episomal form.[13]

Transmission of CMV

Transmission of CMV occurs by acquisition of cell free virus at mucosal sites, by close contact with a person shedding the virus in body fluids including urine, saliva, breast milk, cervical and vaginal secretions, and semen.[7] Particularly urine and saliva of young children may contain high virus titers and are therefore major sources of CMV.[7] No studies have supported transmission of CMV through respiratory droplets. Blood-borne transmission through blood products and organ allografts can occur, and transplacental transmission results in congenital infection (discussed below).[7] Perinatal transmission of CMV in the birth canal and during breast-feeding is common. Up to 96% of CMV seropositive mothers shed CMV (DNA) in mature cell free breast milk at some time during lactation, with a peak excretion between 2 weeks and 2 months postpartum.[7,14] About 40% of the preterm newborns breastfed for at least 1 month by CMV seropositive mothers become infected postnatally.[7,14,15]

Epidemiology of CMV infection

CMV circulates worldwide and is endemic in the whole human population, without seasonal variation. The seroprevalence of CMV increases with age and varies widely depending on ethnic and socioeconomic background. CMV seroprevalence is high in developing countries, up to 95-100% among preschool children in sub-Saharan Africa, South America, and Asia.[7] In contrast, CMV seroprevalence of less than 20% has been found in subpopulations in the United Kingdom and the United States.[7] In women of childbearing age, CMV seroprevalence is above 90% in developing countries, and 40-85% in the United States and Western Europe.[7,16] In the Netherlands, maternal CMV seroprevalence ranges between 41-73% among various subgroups.[17,18] High CMV seroprevalence among populations of low socioeconomic status reflects increased exposure to CMV due to factors including large household size, crowding, certain child care practices, and possibly sexual practices.[7] Day-care centers facilitate transmission of CMV. About half of the infants attending day

care centers with middle- to upper-income background shed CMV in their urine and saliva.[19]

The reproductive number (R_0) of CMV has been estimated using mathematical models based on age-specific seroprevalence data.[20,21] This R_0 is relatively modest, being 1.7-2.4 in Western populations, indicating that an infected person transmits CMV to approximately two susceptible people.[20,21] The R_0 is somewhat higher in subpopulations of low socioeconomic status, up to 4.1 in non-Hispanic blacks in the Unites States.[20,21] Corresponding with this R_0, the force of infection is relatively low and has been calculated as an average annual seroconversion rate of 1.6-2.3% among pregnant women in the United States and the United Kingdom, and 5-20% in subpopulations of low socioeconomic status (Figure 1).[20,22,23]

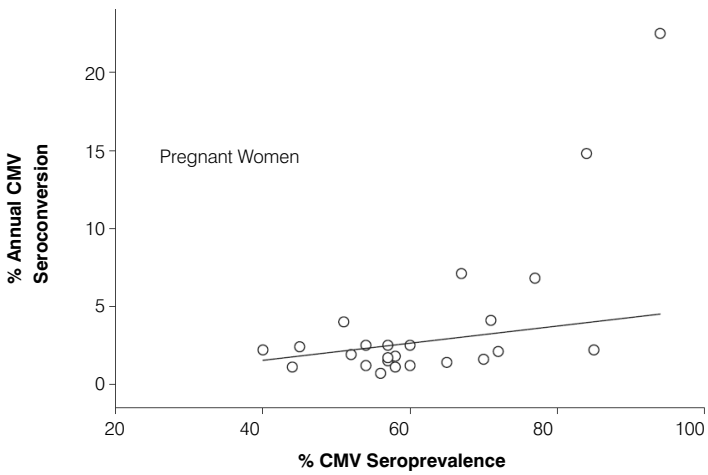


Figure 1 Meta-analysis of studies reporting annual CMV IgG seroconversion rates among pregnant women, as a function of CMV seroprevalence in the underlying population. (Adapted from Hyde et al.[22])

Clinical manifestations in adults

Primary CMV infection in the immunocompetent child or adult has been described as being usually asymptomatic[4], however there are no data on the exact proportion of symptomatic primary infections. Uncommonly, primary infection in the immunocompetent host results in a mononucleosis syndrome clinically similar to the syndrome associated with Epstein-Barr virus infection.[6,24] CMV mononucleosis may account for 8-20% of cases with mononucleosis syndrome presentations.[6,24]

Infrequent complications of CMV mononucleosis include pneumonia, hepatitis, central nervous system involvement (Guillain-Barré syndrome), aseptic meningitis, encephalitis, pericarditis, and myocarditis.[4,6] Most postnatally infected newborns do not develop symptoms[4,25], although occasional cases of severe disease including pneumonitis, hepatosplenomegaly, lymphadenopathy, and aseptic meningitis within the first 3 months of life have been reported.[4,25] Low birth weight (<1500 g) has been described as a risk factor for symptomatic postnatal infection.[4,25] No association of postnatal CMV infection with hearing loss or neurological developmental impairment has been found, though data on the long-term follow-up of postnatally infected (premature) infants are limited.[25] In immunocompromised patients, CMV can cause significant morbidity and mortality due to CMV colitis, hepatitis, encephalitis, pneumonitis and retinitis.

Epidemiology of congenital CMV infection

Intrauterine infection with CMV is thought to result from maternal viremia and associated placental infection.[4,7] In a meta-analysis, intrauterine transmission was estimated to occur in approximately 32% (95%CI 30-35%, range 14-52%) of the pregnant women with primary infection.[16] The maternal-to-fetal transmission risk after primary infection increases with gestational age and has recently been reported to be up to 64-73% in the third trimester.[26,27,28] In contrast, the highest risk of fetal damage (including hearing loss) exists around conception and in the first two trimesters of pregnancy.[28,29,30,31] In contrast to congenital rubella and toxoplasmosis, where intrauterine transmission occurs principally as a result of primary maternal infection, intrauterine transmission of CMV can occur as a consequence of non-primary or recurrent infection, i.e. reactivation of latent virus or re-infection with a new strain. In a meta-analysis of data on the birth prevalence of congenital CMV among the offspring of seropositive women, the pooled risk of maternal-to-fetal transmission following recurrent infection was 1.4% (95%CI 1.1-1.7%) (Figure 2).[16]

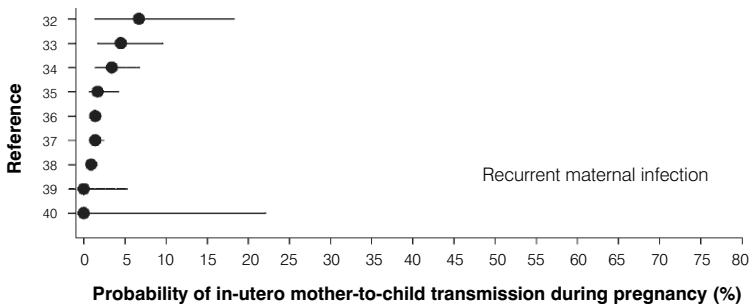


Figure 2 Meta-analysis of studies reporting the prevalence of CMV at birth (congenital infection) among the offspring of CMV seropositive pregnant women, described as the maternal-to-fetal transmission risk following recurrent maternal infection. Lines represent 95%CI. (Adapted from Kenneson et al.[16])

In the same meta-analysis, the combined birth prevalence of congenital CMV reported by 27 worldwide study groups was 0.64% (95%CI 0.60-0.69%), with considerable variability among different populations.[16] Data on the birth prevalence of congenital CMV in the Netherlands are limited (until publication of the work presented in this thesis) to a single study reporting a birth prevalence of 0.09% in a selected sample of newborns with a low proportion of immigrants.[17] In general, the birth prevalence of congenital CMV increases with maternal CMV seroprevalence in the population, with a birth prevalence estimate of about 2% or higher in populations with >95% seroprevalence (Figure 3).[16] Recently, a prospective, serological study has shown that re-infection with new strains occurred in about 8% of seroimmune pregnancies among a population with nearly 100% CMV seroprevalence (Brazil).[41] Risk factors for congenital CMV infection, mainly derived from case-control studies, are summarized in Table 1.

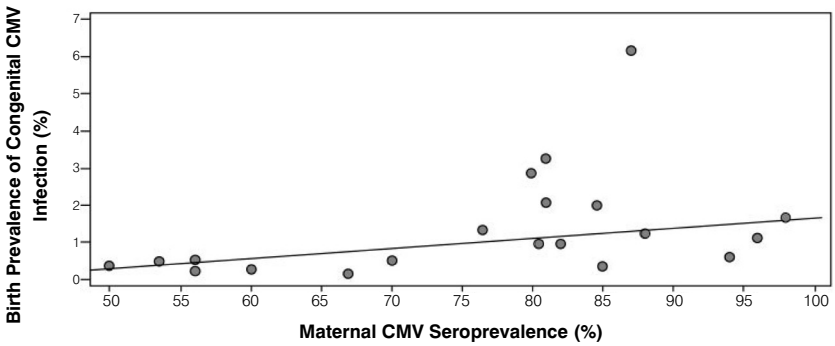


Figure 3 Birth prevalence of congenital CMV is positively correlated with maternal CMV seroprevalence in the population. Each circle represents the birth prevalence estimate from one study group. In lineair regression analysis, every 10% increase in seroprevalence corresponded to a 0.26% increase in birth prevalence. The coefficient of determination (R^2) was 0.29 [16,42], and 0.55 in an earlier report[16,42], indicating that maternal seroprevalence accounted for respectively 29% [16] and 55% [43] of the variability of birth prevalence of CMV between study populations. (Adapted from Kenneson et al.[16])

Table 1 Factors reported to be significantly associated with congenital CMV infection.

Risk factor	Reference
High maternal seroprevalence in the population	16
Non-white race	16
Low socioeconomic status	16
Caring for preschool children	43
Household size >3 people	43
Maternal age <25 years	43
Onset of sexual activity <2 years before delivery	43
STD during pregnancy	43

STD; sexually transmitted disease

Clinical manifestations of congenital CMV

Approximately 10% of the infants born with congenital CMV infection are symptomatic at birth.[16,44] Half of these symptomatic infants present with typical and potentially fatal generalized cytomegalic inclusion disease (CID), characterized by hepatosplenomegaly, microcephaly, jaundice, and petechiae, with or without ocular

and auditory damage.[3,7,45] In total 40-58% of these infants symptomatic at birth will have permanent sequelae.[44] Because early studies focussed on symptomatic infections, congenital CMV was considered a rare and often fatal disease.[7] Nowadays, we realize that 10-15% of the infants born with asymptomatic congenital infection develop neurologic complications throughout the first years of life and will have long-term sequelae.[44] Isolated sensorineural hearing loss (SNHL) is the most common long-term complication of congenital CMV.[46] Because of the late-onset nature of the hearing loss (Figure 4), up to half of the children with congenital CMV-related hearing loss may not be detected in the newborn hearing screening.[47] Among children with hearing loss at later ages, the hearing loss is associated with congenital CMV in 15-40% of the cases.[48,49,50] Other neurologic complications in newborns with congenital CMV are summarized in Table 2, according to symptomatic and asymptomatic status at birth.

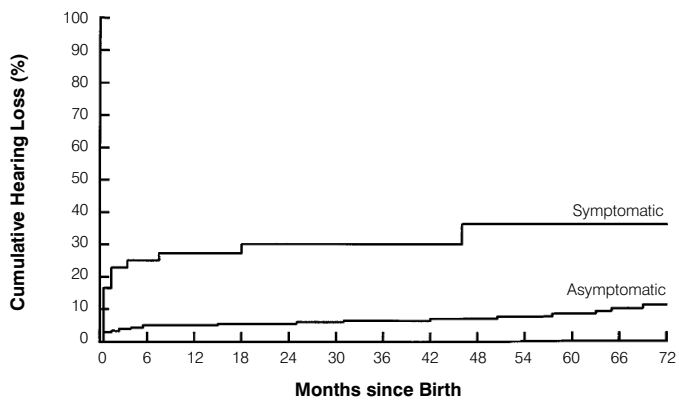


Figure 4 Cumulative hearing loss (>20 dB) in children with congenital CMV infection according to symptomatic and asymptomatic status at birth. (Adapted from Fowler et al.[47])

Table 2 Frequency of neurologic complications in newborns with congenital CMV infection, according to symptomatic and asymptomatic status at birth. (Adapted from Remington and Klein.[7])

	Symptomatic at birth	Asymptomatic at birth
Hearing loss	58%	7%
IQ <70	55%	4%
Chorioretinitis	20%	3%
Seizures	23%	1%
Paresis/paralysis	13%	0%
Death	6%	0%

Clinical symptoms are more frequently seen in newborns from preconceptionally seronegative women[45] (Table 3), indicating that maternal antibodies provide substantial protection against harmful infection in the newborn.

Table 3 Neurologic sequelae in children with congenital CMV infection after primary and non-primary maternal infection. (Adapted from Fowler et al.[45])

	Primary infection	Non-primary infection
Any sequelae	25%	8%
Sensorineural hearing loss	15%	5%
IQ <70	13%	0%
Chorioretinitis	6%	2%
Microcephaly	5%	2%
Seizures	5%	0%
Paresis or paralysis	1%	0%
Death	2%	0%

Postnatal diagnosis of congenital CMV

The gold standard in the diagnosis of congenital CMV is viral culture of urine, sampled within the first 2-3 weeks of life.[51,52,53] After this period, congenital CMV infection cannot be differentiated from the generally harmless postnatally acquired CMV infection.[53] CMV DNA detection (PCR) in urine, saliva, and blood is mentioned in recent literature and described in guidelines as acceptable alternative for diagnosing congenital CMV.[54,55,56] Furthermore, CMV DNA detection in dried blood stored on filter paper (dried blood spots, DBS; Guthrie cards) has become of interest and has the advantage that congenital CMV can be diagnosed retrospectively, e.g. when late-onset hearing

loss becomes manifest.[57,58,59,60,61,62,63] Serological testing for CMV IgM in the newborn lacks adequate sensitivity (range 20%[16,64,65]-70%[66]) and specificity (about 95% [66]) for the diagnosis of congenital infection. Sensitivity of IgM serology in the congenitally infected newborn is hampered by the time-frame between fetal infection and birth, and the immature immune system at the time of infection. CMV IgM antibodies can be detected in only about 25% of the DBS of newborns with congenital CMV.[67]

Postnatal antiviral therapy

Antiviral treatment of congenitally CMV infected newborns with clinically apparent disease is generally accepted[54,68,69,70]. Few studies have addressed the efficacy of antiviral treatment on hearing preservation in newborns with symptomatic and asymptomatic congenital CMV infection (Table 4). Results from 1 RCT show that congenitally infected newborns with central nervous system (CNS) disease benefit from ganciclovir with preserved hearing[71] and recent guidelines include the recommendation of antiviral treatment in this specific group of newborns.[54,56] In addition to preserved hearing, improvement of neuro-developmental status after treatment of newborns with CNS disease has been found.[72] Treatment of asymptomatic congenitally CMV infected newborns is currently not recommended because of limited data on the efficacy in that specific group of newborns.[54,56]

Table 4 Studies on the efficacy of antiviral treatment on hearing preservation in newborns with congenital CMV. Case-reports were excluded.

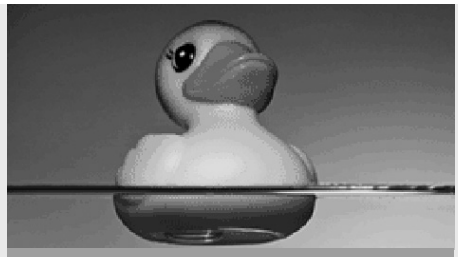
Reference	Newborns	Design	Intervention	Primary outcome	Efficacy	Remarks
Kimberlin et al[71] (2003)	Symptomatic with CNS disease[a] (≤1 mth)	RCT (N=100)	GCV (6 wks)[c]	Hearing[d] at 1 yr	↓ Worsening (P <.01) Treated: 21% Controls: 68% >Efficacy 69%	-50% Lost to follow-up -No placebo, not blinded -Neutropenia Treated: 63%, Controls: 21%
Lackner et al[73] (2009)	Asymptomatic, normal hearing (≤10 dys)	RCT (N=23)	GCV (3 wks)[c]	Hearing[d] at 4-10 yrs	↓ Hearing loss (NS) Treated: 0% Controls: 11%	-No placebo -Neutropenia: 17%
Amir et al[74] (2010)	Symptomatic with CNS disease[a] (≤1 mth)	Retrospective case series, historical controls[71] (N=23)	ValGCV (1 yr)[b,c]	Hearing[d] at 1 yr	↑ Normal hearing (P<.001) 1 yr treated: 76% 6 wks treated: 35%	-Historical controls -Neutropenia: 53%
Baquero-Artigao et al[75] (2011)	Symptomatic with CNS disease[a] (age NR)	Retrospective case series (N=55)	ValGCV (median 6 mths, range 1.4-18 mths)[b,c]	Hearing[d] at 1 yr	Improved: 36% Worsening: 4% (among impaired)	-No controls -Neutropenia: 53%

[a] CNS involvement criteria: (1) microcephaly, (2) intracranial calcifications, (3) abnormal CSF, (4) chorioretinitis, and/or (5) hearing deficits.
[b] Preceded by GCV in 67% [75] to 100% [74] of the patients.
[c] Dosage valGCV: 16 mg/kg twice daily[75], m^3x clearance x 7mg once daily[74], GCV: 5 mg/kg twice daily[74], 10 mg/kg daily[73], 6 mg/kg twice daily[71]
[d] BSER[72,74,75] or OAE[76]

CNS; central nervous system, RCT; randomized controlled trial, GCV; ganciclovir (i.v.), ValGCV; valganciclovir (p.o.), NS; not significant, NR; not reported, CSF; cerebrospinal fluid,
BSER; brainstem-evoked response (=auditory brainstem response, ABR), OAE; otoacoustic emissions

Prevention

Preventive programs for CMV infection have been developed by the United States Centers for Disease Control and Prevention (CDC), and the American College of Obstetricians and Gynecologists (Figure 5). Because exposure to saliva and urine of young children is a major cause of CMV infection among pregnant women[76], it is likely that good personal hygiene can reduce the risk of CMV acquisition. Evidence for the efficacy of hygiene counseling is limited to studies showing a reduced rate of CMV seroconversion of pregnant woman after hygiene counseling.[76,77,78]



Ways a pregnant woman may help reduce her exposure to CMV

- Washing hands frequently with soap and water, especially after changing diapers, feeding a child, wiping a child's nose or drool, or handling children's toys.
- Not sharing cups, plates, utensils, food, or toothbrushes.
- Not sharing towels or washclothes.
- Not putting a child's pacifier in her mouth.
- Cleaning toys, countertops, and anything else that comes in contact with children's urine or saliva.

Figure 5 Hygienic measures recommended by the CDC to pregnant women to reduce the risk of CMV infection in pregnancy (www.cdc.gov/features/dscytomegalovirus/).

Newborn screening for congenital CMV

General criteria for screening have been proposed by Wilson and Jungner (Table 5).[79] In the Netherlands, newborns are routinely screened for 18 metabolic and inherited disorders, including PCR-based screening on cystic fibrosis using DBS (since May 2011). Newborn screening for congenital CMV has only recently been seriously considered, despite earlier appeals for preventive measures for congenital CMV infection.[80,81] The potential for newborn screening for CMV would lie in the identification of the large proportion of asymptomatic congenitally infected newborns at risk for developing late-onset hearing loss. These newborns at risk could benefit from intervention measures such as extensive audiological follow-up and potentially, antiviral therapy.

Table 5 Criteria for screening as proposed by Wilson and Jungner.[79]

The disease	The condition should be an important health problem
	The natural history should be well understood
	There should be a detectable early stage
The screening test	The test should be suitable for the early stage
	The test should be acceptable to patient and staff
	Intervals for repeating the test should be determined
The treatment	Early treatment should be of more benefit than at a later stage
	Adequate health service provision should be made
	The risks should be less than the benefits
	The costs should be balanced against the benefits

Outline of the thesis

The aim of this thesis is to study several aspects of congenital CMV infection in general and more specifically in the Netherlands, in order to determine the necessity and feasibility of newborn screening for congenital CMV. The major topics addressed in this thesis are the following.

PART I

The DISEASE BURDEN of congenital CMV infection in the Netherlands. This topic is addressed in several ways. Firstly, the birth prevalence of congenital CMV in the Netherlands was determined in a cross-sectional study. A large sample of DBS from infants born in the Netherlands was retrospectively tested for CMV DNA (Chapter 2). To address the clinical impact of congenital CMV disease in the Netherlands, the proportion of congenital CMV infections among Dutch children with permanent bilateral hearing loss was determined (Chapter 3). Additionally, to address subpopulations at risk for congenital CMV infection, risk factors for congenital CMV in the Dutch population were analyzed (Chapter 2). Furthermore, maternal immunity to CMV as risk factor for congenital infection was assessed by means of a population-based prediction model (Chapter 4). Finally, awareness of the disease burden of congenital CMV among doctors in the Netherlands was studied using a digital questionnaire sent to doctors involved in mother and childcare. (Chapter 5).

PART II

Postnatal SCREENING TOOLS for congenital CMV were studied by evaluating a large number of DNA extraction methods for dried blood spots (DBS) (Chapter 6 and 7), and by evaluating real-time PCR on urine in the diagnosis of congenital CMV (Chapter 9). Following CMV DNA detection in DBS, the potential to use DBS for genotyping of CMV was assessed (Chapter 8).

PART III

Pros and cons of NEWBORN SCREENING for congenital CMV are summarized and discussed in detail in Chapters 10, 11 and 12. Rationale for potential benefits and disadvantages of newborn screening on congenital CMV are addressed, using the criteria of Wilson and Jungner to summarize the disease burden, the currently available screening tests, and the evidence for intervention options.

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Chapter 2

Congenital cytomegalovirus infection in the Netherlands: birth prevalence and risk factors

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Abstract

Congenital cytomegalovirus (CMV) infection is the most common congenital viral infection worldwide. The sequela encountered most frequently is hearing impairment, affecting approximately one out of five infants congenitally infected. Data on the birth prevalence and risk factors of congenital CMV infection in the Netherlands are scarce. The aim of this study was to determine the birth prevalence of congenital CMV in the Netherlands. A sample of 6,500 dried blood spots (DBS) from infants born in the Netherlands was tested anonymously for CMV DNA. The sample was stratified by the number of live births in different regions of the Netherlands of the year 2007. Additionally, on a regional level, risk factors for congenital CMV were analyzed. The birth prevalence of congenital CMV in the Netherlands was 0.54% (35/6,433, 95%CI 0.36–0.72). Congenital CMV infection was significantly higher in regions with more than 15% young children (0–5 years) compared with regions with a lower proportion of young children (OR 5.9, 95%CI 1.4–25.2). Congenital CMV infection was significantly higher in regions with more than 30% immigrants compared with regions with a lower proportion of immigrants (OR 2.2, 95%CI 1.1–4.6). This association was strongest for regions with more than 30% non-Western immigrants (OR 3.3, 95%CI 1.5–7.5). Based on the knowledge of the natural history of congenital CMV infection, approximately 1,000 children are born with congenital CMV infection in the Netherlands annually, of whom eventually approximately 180 children (0.1% of all newborns) will be affected by long term sequelae, with hearing loss being the symptom encountered most frequently.

Introduction

Cytomegalovirus (CMV) infection is the most common congenital viral infection worldwide. The symptom of congenital CMV infection encountered most frequently is sensorineural hearing loss, which will affect approximately one out of five congenitally infected newborns.[1,2] About 10% of the live-born infants with congenital CMV infection are symptomatic at birth[1,2], whereas an additional 10% of the infected newborns will develop permanent sequelae in the following years.[1-3] Among children with bilateral profound sensorineural hearing loss, the hearing disability is attributable to congenital CMV infection in one out of five patients.[4-6] This makes CMV the leading cause of non-genetic congenital hearing impairment.

The overall birth prevalence of congenital CMV was estimated at 0.64%[2], with significant variety among different countries and populations. The birth prevalence has been shown to be correlated positively with maternal CMV seroprevalence in the population.[2] Established risk factors for congenital CMV infection include preschool children in the household, household size more than three people, non-white race, low socioeconomic status, preconception maternal seronegative status, and maternal age below 25 years.[2,7-9]

Data on the birth prevalence and risk factors for congenital CMV infection in the Netherlands are scarce. Only one study has been published on the birth prevalence of congenital CMV in the Netherlands, estimating a prevalence of congenital CMV of 0.09%.[10] This estimate is low when compared to the birth prevalence estimates from other northern European countries, ranging from 0.18 to 2.0%.[11-16] Furthermore, it is not in accordance with the maternal seroprevalence of CMV in the Netherlands of 41–73%.[20,37] Maternal seroprevalence rates of CMV of 50–70% in other countries have been associated with birth prevalence rates of approximately 0.3–0.6%.[2]

The aim of this study was to determine the birth prevalence of congenital CMV in the Netherlands, in order to estimate the disease burden. A large, random sample of dried blood spots (DBS) was selected from all infants born in the Netherlands in 2007 and analyzed for the presence of CMV DNA. Additionally, the contribution of risk factors for congenital CMV infection was analyzed on a regional level by comparing the birth prevalence of congenital CMV with the demographic characteristics and socioeconomic status parameters of the regions.

Materials and methods

Study design

Of all live newborns in the Netherlands in 2007 (n = 182,765), 99.8% participated in the nationwide metabolic and endocrine screening program for which DBS are collected routinely within a few days after birth[17] and stored at the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands) (room temperature). Of those DBS, a total of 6,500 DBS were selected for this study, stratified for the number of live births per region and the month of birth. Fourteen different regions were identified (12 provinces + Amsterdam + Rotterdam). Testing of the DBS was performed anonymously, thus no information was available on the clinical outcome of these children. The study was approved by the Program Committee Neonatal Screening (RIVM, the Netherlands), and the Medical Ethics Committee (CME) of the Leiden University Medical Center (LUMC, the Netherlands).

CMV DNA Detection in DBS

DNA extraction

DNA was extracted from DBS using the protocol described by Barbi et al.[36] (details obtained by personal communication), as evaluated previously.[18] In short, one punch of 3.2 mm per tube (approximately 3 µl dried blood), in triplicate, was incubated overnight in 35 µl Minimum Essential Medium (Gibco/Life Technologies, Breda, The Netherlands), with a fixed aliquot of phocine herpes virus (PhHV, kindly provided by Bert Niesters, University Medical Center Groningen, the Netherlands) to monitor nucleic acid isolation and PCR inhibition. Incubation was followed by heating for 60 min at 55°C, and for 7 min at 100°C. After cooling, the sample was centrifuged and the supernatant was frozen. After thawing, the DNA extract was used for CMV DNA amplification.

CMV DNA amplification

CMV DNA amplification was performed by means of an internally controlled quantitative real-time PCR as described previously [Kalpoe et al.[40]], with minor modifications.[18] Briefly, 10 µl of DNA extract was added to 40 µl PCR pre-mixture containing CMV and PhHV primers, CMV and PhHV TaqMan probes, $MgCl_2$, and HotStar Master mix (QIAGEN, Hilden, Germany), followed by amplification of a 126-bp DNA fragment of the CMV immediate-early antigen region. Quantification was performed using a dilution series of titrated CMV (Advanced Biotechnologies Inc., Columbia MD, USA) as an external standard.

Interpretation of triplicate PCR results

Interpretation of triplicate PCR results was performed using the flow diagram as proposed by Barbi et al.[22], in which every positive result was confirmed with at least one other positive result. In cases where in the initial test procedure a single positive result was found, a confirmatory PCR procedure including DNA extraction was performed.

Demographic and socioeconomic status characteristics

For analysis of the contribution of risk factors for congenital CMV infection, the postal code numbers (four-digit) of the DBS tested for CMV DNA were retrieved. Demographic and socioeconomic status parameters of the postal code areas of the DBS tested (for the year 2007) were retrieved from Statistics Netherlands (Centraal Bureau voor de Statistiek (CBS), www.CBS.nl/en). Characteristics of the postal code areas of the CMV-positive DBS were compared to the characteristics of the postal code areas of the CMV-negative DBS (analogue to a comparison of the birth prevalence of congenital CMV between regions with and without these characteristics). Demographic characteristics analyzed were (non-Western) immigrants, young children (0–5 years) in the population, and household size. Socioeconomic status characteristics analyzed were income and educational level.

Sample size and statistical analysis

The sample size calculation was based on an estimated birth prevalence of 0.4%, a significance level of 5%, and 80% power. For risk factor analysis, sub-population numbers were expressed as proportions of the total populations in that area (e.g., the number of non-Western immigrants was divided by the total number inhabitants in that area), and categorized. Category cut-offs were based on the distribution of the characteristics in the community, while maintaining sufficient numbers in the contingency table in order to achieve reasonable power. Differences in categorical data were compared with the Chi-square test and the Fisher's exact test (for expected frequencies below 5), and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. Data were analyzed using SPSS version 17.0 (SPSS Inc., Chicago, IL) with P-values < 0.05 (two-sided) considered to be statistically significant.

Results

Birth prevalence of congenital CMV in the Netherlands

Out of 6,500 selected DBS, 6,433 DBS were available for CMV detection. The 67 DBS were not available for testing since parents had not given permission for storage of the DBS at the time of metabolic and endocrine screening. Out of the 6,433 DBS tested, 35 DBS were positive for CMV DNA. This corresponds with a birth prevalence of congenital CMV in the Netherlands of 0.54% (95%CI 0.36–0.72%). The median CMV DNA load was 3.7 $\log_{10}$ (5,012) copies/ml whole blood, with a median cycle threshold of 40 (range 37–44). Fifteen, 7, and 13 of the 35 confirmed CMV positive DBS were initially positive in respectively one, two, and three of the triplicates.

Risk factors for congenital CMV

Postal code numbers were retrievable for 6,022 of the 6,433 DBS, resulting in 2,180 different postal code areas. On average, a postal code area contained 8,261 inhabitants (range 30–27,030). Demographic characteristics of the postal code areas of the 6,022 DBS were retrieved, and data on households/immigrants, income, and education were available for the 5,930, 5,424, and 4,589 DBS, respectively. Demographic characteristics and socioeconomic status parameters of the postal code areas and the prevalence of congenital CMV in those areas are shown in Table 1. Two out of 32 CMV positive DBS were from regions with a population with more than 15% young children (0–5 years), whereas 66 out of 5,898 CMV negative DBS were from regions with a population with more than 15% young children. This is analogous to a birth prevalence of 2/68 (2.94%) in regions with more than 15% young children, whereas the birth prevalence was 5.9 times less prevalent in regions with a lower proportion of young children (30/5,862 (0.51%), OR 5.89, 95%CI 1.38–25.16). Furthermore, congenital CMV was significantly more prevalent in regions with more than 30% immigrants compared to regions with a lower proportion of immigrants (OR 2.20, 95%CI 1.06–4.57). This association was strongest in regions with more than 30% non-Western immigrants (OR 3.33, 95% CI 1.49–7.46), but remained significant when the category cut-off was lowered to more than 20% non-Western immigrants. When analyzed in a multivariate logistic regression model, the variables more than 15% young children in the population (OR 4.46, $P = 0.048$) and more than 30% non-Western immigrants (OR 3.08, $P = 0.007$) remained significantly associated with congenital CMV infection. No significant association was found between the prevalence of congenital CMV and a mean household size of more than 3.0 persons.

Additionally, congenital CMV infection was not found to be significantly associated with regions with a high proportion of households with lower income, a low proportion of households with higher income, or with lower education (Table 1).

Table 1 Demographic and Socioeconomic Status Characteristics of the Postal Code Areas, and the Birth Prevalence of Congenital CMV

Characteristic	Birth prevalence of congenital CMV when characteristic		OR (95% CI)[a]	*P*-value[ab]
	Present	Absent		
Young children				
>15% of population children <5 years	2.94% (2/68)	0.51% (30/5,862)	5.89 (1.38–25.16)	0.007
Immigrants				
>30% Total immigrants	0.96% (11/1,146)	0.44% (21/4,784)	2.20 (1.06–4.57)	0.03
>20% Non-Western immigrants	0.98% (10/1,016)	0.45% (22/4,914)	2.21 (1.04–4.68)	0.03
>30% Non-Western immigrants	1.47% (8/544)	0.45% (24/5,386)	3.34 (1.49–7.46)	0.002
Household size				
Mean household size >3.0 persons	0% (0/132)	0.55% (32/5,798)	0 (-)	0.39[c]
Socioeconomic status				
Income				
>40% Households with income <16 700€	0.66% (5/758)	0.49% (23/4,666)	1.34 (0.51–3.54)	0.55
<20% Households with income 31 900€	1.80% (2/111)	0.49% (26/5,313)	3.73 (0.87–15.92)	0.06[c]
Education				
>45% Households with lower education	0.96% (2/209)	0.43% (19/4,380)	2.22 (0.51–9.58)	0.27[c]

CMV, cytomegalovirus; OR, odds ratio; CI, confidence interval.
[a]Univariate analysis.
[b]Chi-square test unless stated otherwise.
[c]Fisher's exact test.

Discussion

This study shows that the birth prevalence of congenital CMV in the Netherlands is approximately 0.54%. It is the first report on the birth prevalence of congenital CMV testing a large selection of DBS covering all regions of the Netherlands. Given the large sample size and the stratification by the number of births in the different regions, the birth prevalence determined is expected to be valid and representative for the Netherlands as a whole. The birth prevalence found in this study corresponds with the birth prevalence of congenital CMV reported in other northern-European countries (0.18–2.0%)[11-16], where significant differences are found among different (sub)populations. Furthermore, the birth prevalence calculated in this study is in line with the maternal seroprevalence of CMV in the Netherlands of 41–73%[10,19], which has been shown to be correlated positively with the birth prevalence of congenital CMV in a population.[2] Previously, Gaytant et al.[10] described a birth prevalence of 0.09% in the Netherlands. The major drawback of that prospective study was that the newborns studied were born in the south-eastern part of the Netherlands with a probable under-representation of newborns from non-native parents. They found that the seroprevalence of CMV was significantly lower in this area than in the metropolitan area of Amsterdam and Rotterdam. Also, though several studies have shown reasonable sensitivities of 87–100%[20,21] of saliva sampling, the diagnostic approach used by Gaytant et al., consisting of cord blood serology followed by throat swab PCR and subsequently urine culture, may not have been optimal technically. Thus, the birth prevalence number reported by Gaytant et al.[10] is not likely to be representative of the birth prevalence of congenital CMV in the Netherlands.

It is likely that the actual birth prevalence of congenital CMV in the Netherlands is even higher than the number calculated in our study, due to possible suboptimal sensitivity of DBS testing. Analytical and clinical sensitivities of CMV DNA detection reported previously using DBS vary within a wide range from 34% up to 100%.[18,22-33] A small number of prospective studies have analyzed the sensitivity of CMV DNA detection in DBS, testing a large population of unselected newborns in comparison with the gold standard, i.e., urine CMV culture or PCR at 2–3 weeks after birth, and reported sensitivities of 34–83%.[25,26,29,32,34] Using these sensitivities, the actual birth prevalence of congenital CMV in the Netherlands could be as high as 0.65–1.59%. We and others have shown that optimizing DNA extraction protocols, PCR techniques and testing algorithms, e.g., by means of performing independent triplicate testing, increases analytical sensitivity significantly[18,28,32], and the DBS assay used in this study was

optimized previously.[18] Besides above mentioned technical aspects, CMV load in blood has been described to be significantly lower than that in urine[35], which may have affected the detection of CMV in DBS in our study. In addition to the possible suboptimal sensitivity, the CMV status of the 67 DBS without parental permission for storage was not known. These DBS originated from a rural region of the Netherlands referred to as the Bible Belt, containing a low proportion of immigrants, rendering it unlikely that a high number of congenital CMV cases were among these unavailable DBS. The specificity of CMV PCR assays for DBS has been reported to range between 99.3 and 100%.[22,36,37] In the current study, the possibility of false positive test results was minimized by using an optimized test strategy including confirmatory testing of (initial single) positive test results, resulting in a specificity approaching 100%.

This study illustrates that congenital CMV infection is approximately six times more prevalent in those areas in the Netherlands with more than 15% young children in the population compared with areas with a lower proportion of young children. Additionally, we show that congenital CMV infection was more prevalent in areas with a higher proportion of immigrants, with the birth prevalence being three times higher in areas with more than 30% non-Western immigrants compared with areas with a lower proportion of non-Western immigrants. The findings correspond with results from studies assessing risk factors for congenital CMV infection in other countries.[2,7-9] The proportion of young children and immigrants in a population are demographic markers for environmental factors and behaviors that facilitate CMV transmission. Young children shed CMV in their body fluids and are, therefore, a common source for CMV. A CMV shedding child is a known risk factor for maternal CMV infection.[33] Among immigrants, maternal CMV seroprevalence has been shown to be higher than among native mothers in the Netherlands.[10] A higher maternal seroprevalence implies a more frequent exposure to CMV, which may be related to cultural differences in childcare practices (with frequent contact with children's saliva, urine, and maternal breast milk) and/ or sexual activities. Previous studies suggest a positive correlation of congenital CMV with a household size more than three persons and low socioeconomic status.[2,7-9] However, in the present study no significant association between a big household size and congenital CMV infection was found. Due to the anonymized data of the DBS tested, no information was available on the clinical outcome of the children and risk factors could not be assessed at the individual level. Despite the latter limitation, risk factors were analyzed at regional level. Since the sample size was calculated to assess a reliable estimate of the birth prevalence of congenital CMV in the Netherlands, the risk factor analysis had limited power due to

relatively low numbers. It is possible that parameters lacking a significant association in the study, might in fact be significantly associated when analyzed with a larger sample size. Finally, the limited availability of demographic and socioeconomic status variables made that not all risk factors important for congenital CMV infection could be studied.

Based on the knowledge of the natural history of congenital CMV infection[1], a birth prevalence of congenital CMV of 0.54% implicates that approximately 1,000 children are born with congenital CMV infection in the Netherlands annually, of whom approximately 180 children (0.1% of all newborns) will develop long term sequelae (Fig. 1). These long term sequelae include hearing loss, cognitive and/or motor deficits and have significant impact on the lives of patients and their families, rendering congenital CMV infection an important public health problem. The number of children with sequelae due to congenital CMV infection is the same order of magnitude as the total number of newborns detected annually with the newborn hearing screening and metabolic screening programs in the Netherlands.[17] CMV infection is, therefore, an important public health issue warranting further research to assess which preventive measures are most cost-effective.[39]

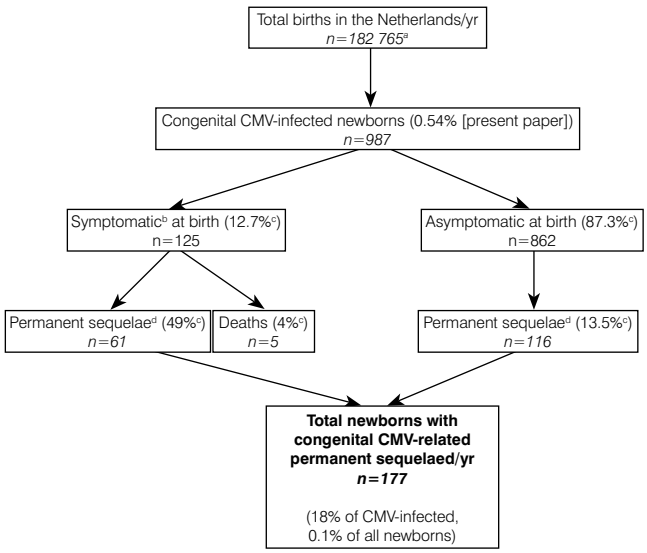

Figure 1 Birth prevalence and implications for disease burden of congenital CMV in the Netherlands.
[a] Lanting et al.[17]
[b] Symptomatic: Petechiae, jaundice, hepatosplenomegaly, thrombocytopenia, chorioretinitis, seizures, microcephaly, intracranial calcifications, or fetal hydrops.[1]
[c] Dollard et al.[1]
[d] Sequelae: Sensorineural hearing loss (uni-and bilateral), cognitive deficit (mental retardation, neurological impairment and developmental delay), and motor deficit.[1]

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Chapter 3

DECIBEL study: congenital cytomegalovirus infection in young children with permanent bilateral hearing impairment in the Netherlands

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Abstract

Background

A significant number of asymptomatic newborns infected with congenital cytomegalovirus (CMV) will present with permanent childhood hearing impairment (PCHI) during early childhood.

Objectives

To investigate the role of congenital CMV infection in causing PCHI in the Netherlands, and assess the efficacy of two different hearing screening strategies and the developmental outcome following each strategy.

Study design

We included 192 children with PCHI at the age of 3–5 years, who were offered hearing screening in their first year of life. Dried blood spots from 171 children were available for CMV detection using real-time PCR. The results of eight previously tested samples were also available. Clinical baseline characteristics were collected from medical records and the Child Development Inventory was used to investigate the developmental outcome.

Results

The rate of congenital CMV among the 179 children was 8% (14/179) and 23% (9/39) among children with profound PCHI. Two of eight CMV-positive children with PCHI at the age of 3–5 years had passed the newborn hearing screening (NHS) test. Developmental outcome measures showed a significantly greater delay in language comprehension in children with both PCHI and congenital CMV infection (the largest in symptomatic children) than in the children with PCHI without congenital CMV infection.

Conclusions

Congenital CMV infection is important in the etiology of PCHI. Universal NHS is not a guarantee of normal hearing and development in childhood for children with congenital CMV infection. This is a problem which might be solved by universal congenital CMV screening.

Introduction

The leading non-genetic cause of permanent childhood hearing impairment (PCHI) is congenital cytomegalovirus (CMV) infection.[1] Approximately 85% of infants with congenital CMV infection do not exhibit signs or symptoms at birth, but about 15% of these children will develop permanent sequelae, such as PCHI and general developmental delay, during early childhood.[2–4] Isolated PCHI is the most frequent long-term sequel in children with congenital CMV infection.[5,6] To diagnose congenital CMV later in life, e.g. in children presenting with PCHI, stored dried blood spots from blood drawn within the first week of life can be used for CMV DNA detection. This is a practical and reliable method for diagnosing congenital CMV infection later in life, since dried blood spots can be stored for very long periods without loss of sensitivity.[7]
It has been suggested that newborn hearing screening (NHS) may fail to detect children with progressive or delayed-onset hearing loss linked to congenital CMV infection.[8] Since 2002, infant hearing screening using distraction methods has been gradually replaced by universal newborn hearing screening (NHS) in the Netherlands. One of the aims of our study was to determine the efficacy of the NHS program in relation to congenital CMV-related hearing loss.
Furthermore, little is known about the developmental outcome of children with both PCHI and congenital CMV. Previous studies on the developmental outcome of children with congenital CMV infections show marked heterogeneity.[3]

Objectives

The aim of the present study was to investigate the contribution of congenital CMV infection to PCHI in children in the Netherlands. Furthermore, the efficacy of two hearing screening strategies and the developmental outcomes that followed these were determined.

Study design

The contribution of congenital CMV to causing PCHI, and its consequences for hearing screening strategies and child development were studied within the framework of the DECIBEL study. The current sub-analysis included all children for whom congenital CMV results were available.

DECIBEL study

The DECIBEL study is a pseudo-randomized study investigating the effects of two different hearing screening strategies on the development of children with PCHI. The development of 3-, 4- and 5-year-old children with PCHI, who were offered either the distraction hearing screening strategy (DHS, at 9 months) or the NHS (within 2 weeks of birth), was evaluated.

The NHS program for healthy newborns, fully implemented in 2006, has a national coverage of approximately 98%, and is a threestep screening program. The first step uses otoacoustic emission testing. In the case of absent emissions in one or both ears this procedure is repeated once. This is followed by automated auditory brain stem response (A-ABR) testing when an abnormal result persists. Referral for extensive audiological diagnostic evaluation follows when these three steps fail to produce a normal screening result. Early hearing screening for infants admitted to a neonatal intensive care unit is carried out using three-step A-ABR testing. Since NHS has gradually replaced DHS in the Netherlands from 2002 onwards, approximately half of the children available for participation in the DECIBEL study have been tested by NHS and the other half by DHS. Diagnostic investigations for congenital CMV infection took place in the workup to determine the etiology of the PCHI in children in the DECIBEL study.

Study population

The study population consisted of children born in the Netherlands between January 2003 and December 2005, who were offered hearing screening in the first year of life and were known to have PCHI at the age of 3, 4 or 5 years at any of the Dutch Audiological Centers. PCHI was defined as a hearing loss of ≥40 dB in the better ear. The children were identified at the participating Audiological Centers. To date, 192 children eligible for participation have taken part in the developmental and etiological assessments of the DECIBEL study, of whom 188 gave informed consent for congenital CMV detection using their dried blood spots. These were not available for 17 children, but it was known that 8 of these had previously been tested for congenital CMV by other institutions. This resulted in 171 dried blood spot cards available for testing, and a total of 179 available results.

Participating children were classified as symptomatic for congenital CMV infection when one of the following conditions was present at birth: intrauterine growth retardation, microcephaly, prolonged hyperbilirubinemia, thrombocytopenia, petechiae or hepatosplenomegaly.

Study specimens and specimen processing

In the Netherlands a blood sample is routinely taken from all newborns during the first week of life for screening for metabolic, endocrine and other disorders. The remaining blood is stored for 5 years as dried blood spots on Guthrie cards by the National Institute for Public Health and Environment (RIVM). The Guthrie cards from the children participating in the DECIBEL study could be recalled with written parental permission. Medical and audiological files, including hearing screening strategies, and correspondence from medical specialists, were used to characterize the study population.

DNA extraction from dried blood spots was performed according to the method described by Barbi et al.[9] using one 3.2 mm punch, as evaluated by de Vries et al.[10] Extraction was performed in 96well plates, and was followed by amplification of a 126-bp fragment from the CMV immediate-early antigen region by means of an internally controlled quantitative real-time polymerase chain reaction as described previously by Kalpoe et al.[11] Each sample was tested in triplicate with a negative control punch between each sample. The results of the triplicates were interpreted using the algorithm described by Barbi et al.[12] The parents of the participants, and their family doctors, were personally informed about the results of the CMV DNA detection.

Assessment of development

The Minnesota Child Development Inventory was translated, according to the rules formulated by Guillemin et al., into the Dutch language and adjusted for sign language (CDI-NL).[13] This parental questionnaire consisted of 270 yes or no statements on child behavior and development, and was sent to parents of participating children by mail or e-mail. The developmental items were grouped to form scales including social development, self-help, gross and fine motor development, expressive language and language comprehension. The general development score was a summary score that provided an overall index of development. The developmental quotients (GDQ, general development quotient; ELC, expressive language quotient; LCQ, language comprehension quotient) were derived using the developmental age divided by the chronological age, and multiplying the result by 100.

Data analysis

The prevalence of congenital CMV among children with PCHI was calculated. Statistical tests were carried out using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA), with the significance level set at $P < 0.05$. The χ^2-test was used to compare the differences in baseline characteristics. Linear regression modeling was used to analyze the developmental outcome based on the CDI-NL. Adjustment was made for age at examination and severity of hearing loss.

Results

Contribution of congenital CMV to PCHI

CMV DNA was detected in 10 of the 171 dried blood spots tested during this study. When the eight children who had been tested previously, but who could not be retested because of missing dried blood spots (four positive and four negative) were added, the total contribution of congenital CMV infection in young children with PCHI was 8% (14/179). Twenty-three percent (9/39) of all cases of profound PCHI (hearing loss $>$ 90 dB) in this sample were attributable to congenital CMV infection. The baseline characteristics of the children with congenital CMV, and those without congenital CMV are presented in Table 1.

No significant differences were found between the two groups in the baseline characteristics of gender, ethnicity, gestational age, type of hearing screening strategy and parity of the mother. The degree of hearing loss was more severe, and progression of hearing loss was significantly more frequent in children with congenital CMV infection than in children without congenital CMV. Additionally, children with congenital CMV infection had received cochlear implants significantly more frequently than children without congenital CMV infection.

Table 1 Baseline characteristics of the study population: children with permanent childhood hearing impairment with and without a congenital cytomegalovirus infection.

	PCHI with congenital CMV n = 14 (%)	PCHI without congenital CMV n = 165 (%)
Gender		
Male	5 (35.7)	100 (60.6)
Female	9 (64.3)	65 (39.4)
Ethnicity		
Caucasian	13 (92.9)	139 (84.2)
Mediterranean/African/Asian	0	10 (6.1)
Mixed	1 (7.1)	12 (7.3)
Unknown	0	4 (2.4)
Gestational age		
25–30 weeks	0	11 (6.6)
31–37 weeks	2 (14.3)	30 (18.2)
≥38 weeks	12 (85.7)	122 (74.0)
Unknown	0	2 (1.2)
Parity		
Primi-para	2 (14.3)	53 (32.2)
Multi-para	12 (85.7)	102 (61.8)
Unknown	0	10 (6.0)
Type of hearing screening strategy		
Distraction hearing screening	4 (28.6)	44 (26.7)
Newborn hearing screening	8 (57.1)	109 (66.0)
Audiological examination on indication	0	7 (4.3)
No hearing screening	2 (14.3)	4 (2.4)
Unknown	0	1 (0.6)
Severity of hearing loss*		
Moderate (40–60 dB)	3 (21.4)	90 (54.5)
Severe (60–90 dB)	2 (14.3)	44 (26.7)
Profound (>90 dB)	9 (64.3)*	30 (18.2)
Unknown	0	1 (0.6)
Progression of hearing loss*	6 (43.0)*	33 (20.0)
Type of amplification*		
Hearing aid	5 (35.7)	117 (70.9)
Bone-anchored hearing aid	0	9 (5.5)
Cochlear implant	8 (57.1)*	33 (20.0)
No hearing aid	1 (7.2)	5 (3.0)
Unknown	0	1(0.6)

* $p < 0.05$.

Hearing screening in children with symptomatic and asymptomatic congenital CMV infection at birth

The hearing screening history and the long-term characteristics of children with congenital CMV infection are shown in Table 2. Four children had been screened by DHS, of whom two passed. Two children born in a region where DHS was offered did not take part in the hearing screening program. Eight children had been screened by NHS, of whom two (symptomatic children) passed. These children presented for audiological evaluation at 27 and 51 months, respectively, because of parental concern. One of them presented with profound hearing loss. Six children had symptomatic disease, of whom two were recognized at birth as having congenital CMV infection. These two children both had a referral at hearing screening and were treated for their symptoms. One received antiviral therapy (ganciclovir 12 mg/kg/day intravenously for 5 weeks and 1 week oral therapy). All children with symptomatic disease at birth had profound PCHI at the age of 3–5 years.

Cerebral imaging had been previously performed in eight children; in three because of multidisciplinary workup to determine the cause of PCHI, in one because of cochlear implant candidacy, and in four because of the suspicion of congenital infection in childhood. Abnormalities that could be interpreted as being caused by congenital CMV infection were seen in six children.

Table 2 Characteristics of the children with permanent childhood hearing impairment and congenital cytomegalovirus (CMV) infection.

	Children positive for congenital CMV infection[a] (n = 14)	
Type of hearing screening offered and result of screening	Distraction hearing screening	
	Refer	2 (1)
	Pass	2 (0)
	Newborn hearing screening	
	Refer	6 (2)
	Pass	2 (2)
	No screening	2 (1)
Reason for audiological evaluation	Refer at hearing screening	7 (4)
	Parental concern[b]	7 (2)
Reported long-term effects	Motor delay	6 (4)
	Visual impairment	12 (2)
	Cognitive delay	3 (3)

[a] Figures in brackets are children with symptomatic infection at birth.
[b] One of these children refused audiological evaluation after referral following distraction hearing screening, but presented later in childhood because of parental concern.

Developmental outcome

CDI-NL results were available from 158 children with PCHI (Table 3). The presence of congenital CMV infection was accompanied by lower raw mean developmental quotients. These scores were even lower for children with symptomatic infection. There was a significant difference in ELQ and LCQ between children with and without congenital CMV infection, in favor of the children with PCHI without congenital CMV infection. Adjustment for age at developmental evaluation or for the severity of hearing loss did not add to the results. Among the children with congenital CMV, a developmental difference, although not significant, was found between asymptomatic children and children with symptoms at birth. In these children, adjustment for age and severity of hearing loss decreased the mean difference. The one child treated with antiviral therapy did not perform better than the untreated CMVpositive children.

Table 3 The mean developmental quotients for children with permanent childhood hearing impairment (PCHI) with or without cytomegalovirus (CMV) infection, and the differences in quotient points between children with PCHI with or without CMV infection (asymptomatic or symptomatic at birth).

	General development quotient	Expressive language quotient	Language comprehension quotient
Without congenital CMV infection	77.7	79.3	71.4
With congenital CMV infection	67.4	62.7	56.4
Asymptomatic at birth (acCMV)	69.7	66.2	62.4
Symptomatic at birth (scCMV)	64.3	58.1	48.4
Mean difference (β) between children with and without congenital CMV (95% CI)	−10.4 (−21.2; 0.5)	−16.6 (−32.6; −0.5)*	−15.0 (−29.2; −0.8)*
Mean difference (β) between children with and without congenital CMV (95% CI)[a]	−9.6 (−20.4; 1.2)	−15.0 (−30.7; 0.7)	−14.2 (−28.4; −0.01)*
Mean difference (β) between children with scCMV and children without congenital CMV (95% CI)	−13.4 (−29.5; 2.7)	−21.2 (−45.2; 2.7)	−23.1 (−44.3; −1.8)*
Mean difference (β) between children with scCMV and children without congenital CMV (95% CI)[a]	−12.7 (−28.8; 3.4)	−20.3 (−43.8; 3.2)	−22.7 (−43.8; −1.6)*
Mean difference (β) between children with scCMV and acCMV (95% CI)	−5.4 (−30.3;19.6)	−8.1 (−39.1;22.9)	−14.1 (−46.9;18.7)
Mean difference (β) between children with scCMV and acCMV (95% CI)[a]	−2.4 (−26.5; 21.7)	−3.0 (−31.0; 25.0)	−14.4 (−47.1; 18.3)

* $p < 0.05$.

[a] Adjustment made for age, and severity of hearing loss.

Discussion

The prevalence of congenital CMV infection in young children with PCHI found in this study was 8%. In children with profound PCHI the prevalence of congenital CMV was 23%. Children with congenital CMV infection are at risk for PHCI, even if they have a normal hearing result at NHS, and the developmental outcome of children with PCHI is significantly negatively affected by the presence of congenital CMV infection.

When interpreting the results, some advantages and a few potential weaknesses of this study need to be taken into account. The 5-year storage of dried blood spots in the Netherlands provided us with the opportunity to diagnose congenital CMV retrospectively.[14] Long-term sequelae of congenital CMV, such as PCHI, had time to become apparent in the intervening years. In the absence of systematic hearing screening in the preschool years, moderate hearing losses may have gone unnoticed, leading to underestimation of the prevalence of PHCI, and underestimation of the overall contribution of congenital CMV infection to PCHI in this study.

The sensitivity of CMV DNA detection in dried blood spots is limited, with sensitivities reported ranging from 50% (for dried blood spots with CMV DNA loads of 3–2 $\log_{10}$ copies/ml) to 100% (for dried blood spots with CMV DNA loads of 5–4 $\log_{10}$ copies/ml) when using the most sensitive methods.[9,10,15,16] Therefore, the contribution of congenital CMV to PCHI found in this study might be underestimated. The underrepresentation of ethnic minorities (non-whites in the DECIBEL study 15%; in the Netherlands as a whole 20%), in whom congenital CMV infection is found more frequently, might be a bias in our study.[17,18] A second possible bias may have been introduced by the urge of parents to gain insight in the etiology of their child's PCHI, leading to a possible overrepresentation of children with PCHI of unknown cause. Only 54% of parents were aware of an underlying cause of the PCHI at the start of the DECIBEL study. The potential (co-)existence of genetic causes of PCHI in these children is the subject of further study. Finally, the limited sample size of children identified with congenital CMV infection is of importance with respect to the interpretation of the results on developmental outcome.

In our study population, bilateral PCHI was attributable to congenital CMV in 8% of cases, and in 23% of children with profound PCHI. Evidence on the contribution of congenital CMV infection to PCHI has been minutely studied by Grosse et al.[19] Reported figures vary between 15% and 40%[20–23]; the fraction of 23% found in the children with bilateral profound PCHI is in concordance with these studies. The prevalence of congenital CMV in children with PCHI reflects the prevalence of

congenital CMV in the country of the study. We expect the prevalence of congenital CMV infection in the Netherlands (0.6–0.7%) to be lower than the estimated overall international prevalence of congenital CMV (0.64%), but the exact prevalence in the Netherlands is unknown to date.[3,14]

Hearing loss caused by congenital CMV might be apparent at birth, but very often it presents during the first years of life.[8] In our study, two children with congenital CMV passed NHS, probably because of delayed-onset or progressive hearing loss. The Joint Committee on Infant Hearing suggests additional hearing evaluations in children with congenital CMV.[24] One should be aware that, lacking universal screening for congenital CMV infection, many congenitally infected children with delayed-onset or progressive hearing loss may be missed by NHS.[25,26]

PCHI in children is expected to lead to a delayed developmental outcome.[27,28] Only a limited number of earlier studies have described the developmental outcome in children with congenital CMV infection, who are considered to be at substantial risk of developmental delay, regardless of auditory involvement.[29,30] The results of our study show that children with PCHI caused by congenital CMV show lower developmental quotients than children with PCHI without congenital CMV. The difference in language development is significant. The raw differences in the language development quotients between children with PCHI with and without congenital CMV infection are large (15 for comprehension and 16.6 for expression). The significant difference in the comprehension quotient persisted when corrected for age and the severity of hearing loss. Further research is necessary to identify possible factors contributing to these results, such as cerebral damage resulting from congenital CMV infection. We recommend that it would be good clinical practice to regularly assess the development of children with congenital CMV, so necessary interventions may be started as soon as possible.

In conclusion, congenital CMV infection is important in the etiology of PCHI. Universal NHS is not a guarantee of normal hearing and development in childhood for children with congenital CMV infection. This is a problem which might be solved by universal congenital CMV screening. Subsequent audiological follow-up of those children with congenital CMV infection could decrease the developmental delay caused by later diagnosis and intervention.

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Chapter 4

The paradox of maternal immunity as a risk factor for congenital cytomegalovirus infection: a population-based prediction model

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Abstract

Background

Maternal immunity to cytomegalovirus (CMV) provides substantial protection against severe congenital CMV disease. Paradoxically, the prevalence of congenital CMV infection increases with CMV seroprevalence in the underlying population.

Objective

To quantify the contribution of non-primary maternal CMV infection on the disease burden of congenital CMV as a function of the seroprevalence in the population.

Methods

A population-based prediction model was developed and applied for a wide range of CMV seroprevalence. Main outcome measures were: the estimated proportion of children with congenital CMV and CMV-related sequelae attributable to non-primary maternal infection, with CMV seroprevalence in the population as independent variable, and the risk for preconceptionally seropositive pregnant women of having a congenitally-infected newborn, compared to this risk for seronegative pregnant women, as a function of the seroprevalence.

Results

Both the proportion of newborns with congenital CMV infection and the proportion of newborns with sequeale, attributable to non-primary maternal infections increased with CMV seroprevalence in the underlying population. These proportions ranged up to 96% (95%CI 88-99%) and 89% (95%CI 26-97%), respectively, in populations with seroprevalence of 95%.

Furthermore, seropositive pregnant women were found to be at higher risk of having a congenitally infected newborn than seronegative pregnant women, for all population CMV seroprevalence values. In contrast, seropositive pregnant women were at lower risk of having a newborn with sequelae related to congenital CMV than seronegative pregnant women.

Conclusions

Our data stress the impact of non-primary congenital CMV infection on the disease burden of congenital CMV, among all (sub)populations. Awareness of the risk for seropositive women of having a newborn with CMV-related sequelae will have significant consequences for preventive strategies including hygiene counseling, maternal serological screening, and immunization studies.

Background

Congenital cytomegalovirus (CMV) infection is an important public health problem with approximately 7 in 1,000 newborns affected.[1] Approximately one in five congenitally infected infants will suffer from long-term neurological sequelae, with hearing impairment being encountered most frequently.[2] Primary maternal CMV infection during pregnancy is transmitted to the fetus in 32 percent of the cases, whereas the transmission risk in CMV seropositive women is about 30-fold lower.[1] Moreover, severe symptoms at birth and long-term sequelae are seen more frequently among congenitally infected newborns from preconceptionally CMV seronegative than seropositive women[2], indicating that acquired maternal immune response provides substantial protection against harmful infection in the newborn. Thus, preventive measures for congenital CMV have mainly been focused on preconceptionally seronegative women.

Paradoxically, a positive correlation between the birth prevalence of congenital CMV and CMV seroprevalence in the underlying population has been found, with birth prevalence ranging from 0.3% to 2% or higher in (sub)populations with CMV seroprevalence of 30% to 98%.[1;3;4] Recent calculations addressed the contribution of non-primary maternal CMV infections to the number of congenital CMV infections in the United States[5] and demonstrated their non-negligible impact. The precise effect of the CMV seroprevalence in the underlying population on the proportion of congenitally infected children with sequelae born to seropositive mothers is largely unknown.

To quantify the contribution of non-primary maternal CMV infection on the disease burden of congenital CMV as a function of the seroprevalence in the population, a prediction model was developed, and applied for a wide range CMV seroprevalence.

Methods

A population-based prediction model was developed, estimating the proportion of children with congenital CMV infection and CMV-related sequelae for non-primary and for primary maternal infection, with seroprevalence in the underlying population as an independent variable. After development, the model was applied for a wide range of CMV seroprevalence.

Model development

The proportion of children with congenital CMV infection and CMV-related sequelae in a population, as a function of the seroprevalence, was estimated as the sum of the proportion of newborns with congenital CMV infection and CMV-related sequelae from seropositive women and from seronegative women (Figure 1). The risk of seropositive pregnant women of having a newborn with congenital CMV infection was composed of the maternal-to-fetal transmission rate in seropositive women. The risk of having a newborn with congenital CMV infection and CMV-related sequelae for seronegative women was composed of the product of the rate of seroconversion during pregnancy and the maternal-to-fetal transmission rate after primary maternal infection. Parameters in this model were based on sero-survey data in the literature, and were estimated as follows.

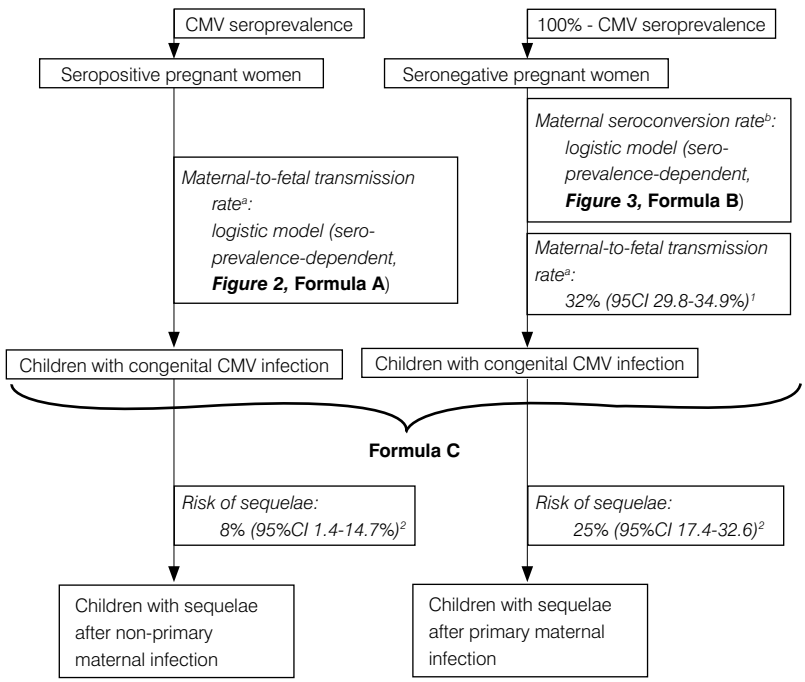

Figure 1 Flow diagram summarizing the model used in this study, estimating the number of children with congenital CMV and CMV-related sequelae, as a function of the seroprevalence in the underlying population, classified by maternal preconceptional CMV IgG seroimmune status.
[a]; per pregnancy, [b]; per year

Maternal-to-fetal transmission rate in seropositive women

The maternal-to-fetal transmission rate in seropositive women, as a function of the seroprevalence in the underlying population, was estimated by performing an analysis of the raw data from reports on the CMV birth prevalence among preconceptionally CMV IgG seropositive women[6-12], listed in a meta-analysis by Kenneson et al.[1] We combined these birth prevalence data with CMV seroprevalence data from the original reports. Only reports with seroprevalence data representative for the underlying population were included (N=7, Figure 2).[6-12] We fitted a logistic regression model on these data-points (curved line) and included random effects to account for heterogeneity between the studies, computing

$$P(\text{CMVnewborn} \mid \text{seropos.pregnancy}) = \frac{1}{1+\exp(6.15-2.44\times\text{seroprevalence})}$$

(Formula A)

In this logistic regression model, CMV seroprevalence was a predictor of the birth prevalence among newborns from seropositive mothers (p=.067, χ^2 test, two-sided).

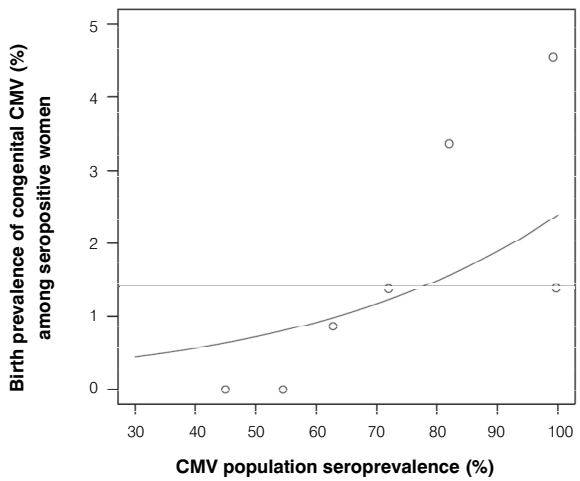

Figure 2 Birth prevalence of congenital CMV among preconceptionally CMV IgG seropositive women (%), as a function of CMV seroprevalence in the underlying population, for each study group.[6-12] The curved line is our logistic regression fit. Each circle represents a study group, previously listed by Kenneson et al.[1]

Maternal seroconversion rate

To quantify the effect of CMV seroprevalence in the underlying population on the seroconversion rate during pregnancy, we fitted a logistic regression model on the combined raw data of the studies listed in meta-analysis by Hyde et al[13] and Wang et al[5] (Figure 3). Hyde et al[13] analysed studies with data on annual CMV seroconversion rates among pregnant women combined with CMV seroprevalence in the study population (N=24 data points).[10-12;14-32] Wang et al[5] reported data on annual CMV seroconversion rates combined with CMV seroprevalence data among several ethnic subgroups in the United States (N=12), extracted from Colugnati et al.[33] We fitted a logistic regression model on these data-points (curved line) and included random effects to account for heterogeneity between the studies, computing

$$P(\text{Maternal seroconversion}) = \frac{1}{1+\exp(6.54 - 3.83 \times \text{seroprevalence})}$$

(Formula B)

In this logistic regression model, CMV seroprevalence was a significant predictor of the birth prevalence among newborns from seronegative mothers ($p<.0001$, χ^2 test, two-sided).

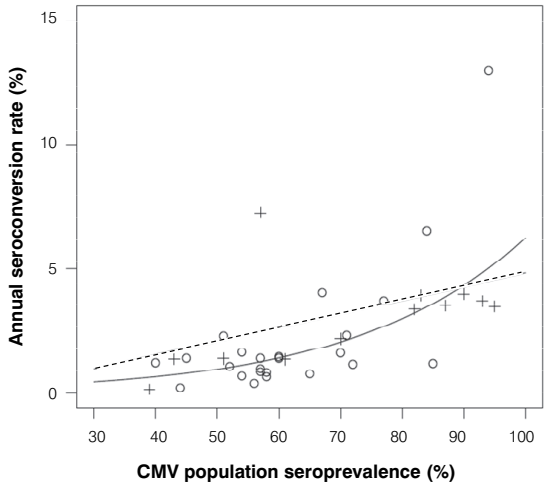

Figure 3 Annual seroconversion rates (%) among seronegative pregnant women, as a function of CMV seroprevalence in the underlying population, for each study group. The curved line is our logistic regression fit, the straight line represents the linear fit of Hyde et al.[13] Circles represent data from studies reported by Hyde et al[13], crosses represent data from subpopulations reported by Wang et al[5].

Maternal-to-fetal transmission rate

The maternal-to-fetal transmission rate following seroconversion was estimated in a previous meta-analysis by Kenneson et al[1] as 32% per pregnancy (95% confidence interval (CI) 29.8%-34.9%).

Overall proportion of congenital CMV

The overall proportion of children with congenital CMV in a population, as a function of the seroprevalence, was the sum of the proportion of newborns with congenital CMV from non-primary and from primary maternal infections, including the proportion of seropositives and seronegatives in the population (seroprevalence and 1-seroprevalence, respectively), resulting in

$$P(\text{CMV newborn} \mid \text{population}) = \frac{\text{seroprevalence}}{1+\exp(6.15 - 2.44 \times \text{seroprevalence})} + \frac{0.32 \times (1 - \text{seroprevalence})}{1+\exp(6.54 - 3.83 \times \text{seroprevalence})}$$

(Formula C)

Risk of sequelae related to congenital CMV

The risk of having a newborn with sequelae related to congenital CMV as a function of the seroprevalence was estimated by supplementing the model with the risk of sequelae following non-primary and primary maternal infection (8% and 25%, respectively[2]). Congenital CMV-related sequelae were defined as sensorineural hearing loss, IQ ≤70, chorioretinits, microcephaly, seizures, paresis or paralysis, and death.[2]

Model application

To quantify the effect of the CMV seroprevalence in the underlying population on the contribution of non-primary maternal infection, the developed model was applied for a wide range of (worldwide present) CMV seroprevalence (30-95%). Outcome measures were the number and proportion newborns with congenital CMV and CMV-related sequelae, and the relative risk for seropositive women.

Newborns with congenital CMV

For CMV seroprevalence 30-95%, the number of children with congenital CMV per 10,000 births for non-primary infection was estimated (10,000 x seroprevalence x Formula A) and for primary infection (10,000 x (1 - seroprevalence) x Formula B x 0.32).

Additionally, for CMV seroprevalence 30-95%, the proportion (%) of children with congenital CMV attributable to non-primary and primary maternal infection,

relative to the total number of children with congenital CMV was estimated ($\frac{\text{Seroprevalence x Formula A}}{\text{Formula C}}$ and $\frac{(1-\text{Seroprevalence}) \text{ x Formula B x } 0.32}{\text{Formula C}}$, respectively).

Sequelae related to congenital CMV

The number and proportion of children with sequelae attributable to non-primary and primary infection, for CMV seroprevalence 30-95%, was estimated by supplementing the estimates of the number and proportion of newborns with the risk on sequelae described above.

Relative risk for seropositive women

For CMV seroprevalence 30-95%, the risk (relative risk, or risk ratio, RR) for preconceptionally seropositive pregnant women of having a newborn with congenital CMV and CMV-related sequelae estimated, relative to this risk for seronegative pregnant women ($\frac{\text{Formula A}}{\text{Formula B x } 0.32}$ and $\frac{\text{Formula A x } 0.08}{\text{Formula B x } 0.32 \text{ x } 0.25}$, respectively).

Sensitivity analysis

Simultaneous 95% confidence intervals (CI) were computed using Monte Carlo simulations[34] (10,000 runs) in which all parameters (maternal-to-fetal transmission rate for seropositive and for seronegative women, maternal-to-fetal transmission rate, and risk on sequelae after non-primary and primary maternal infection) were varied simultaneously. Single-point estimates were selected for each parameter from the respective probability distributions for each evaluation run. 95%CIs, incorporated the uncertainty surrounding each variable.

All statistical analysis were conducted using R (version 2.11.1).

Results

Model application

Newborns with congenital CMV

The estimated number and proportion of newborns with congenital CMV attributable to non-primary and for primary maternal infections, for CMV seroprevalence 30-95% in the underlying population, is shown in Figures 4 and 5.

For example, in a population with 50% CMV seroprevalence, 36 newborns with congenital CMV per 10,000 births were estimated to be attributable to non-primary infections (Figure 4A) and 15 newborns with congenital CMV were attributable to primary infections (Figure 4B). This results in a birth prevalence of congenital CMV of

51 per 10,000 births. The proportion of congenital CMV attributable to non-primary infections in that population is 70% (36 per 10,000 /51 per 10,000 births) (Figure 5A). The estimated number of newborns with congenital CMV attributable to non-primary maternal infections increased with CMV seroprevalence, and ranged from 13 (95%CI 1-54) to 202 (95%CI 82-345) per 10,000 births for seroprevalence of 30% to 95% (Figure 4A). In contrast, the number of newborns with congenital CMV attributable to primary maternal infections ranged from 10 (95%CI 4-16) to 8 (95%CI 4-13) per 10,000 births for seroprevalence of 30% to 95% (Figure 4B).

The proportion of newborns with congenital CMV attributable to non-primary maternal infections increased with CMV seroprevalence, and ranged from 57% (95%CI 24-85%) to 96% (95%CI 88-99%) in populations with CMV seroprevalence of 30% to 95% (Figure 5A).

Sequelae related to congenital CMV

In a similar way, the estimated number and proportion of newborns with sequelae related to congenital CMV (including sensorineural hearing loss) for CMV seroprevalence 30-95% in the underlying population are shown in Figures 4C/D and 5B.

For example, in a population with 50% CMV seroprevalence, 43% of the congenital CMV infections with sequelae were attributable to non-primary maternal infections (3 infected newborns born to seropositive women per 10,000 births, out of in total 7 congenitally infected newborns per 10,000 births).

Both the number and proportion of congenitally infected children with CMV-related sequelae attributable to non-primary maternal infections increased with CMV seroprevalence in the underlying population. The estimated number of children with sequelae attributable to non-primary infections ranged from 1 (95%CI 0-4) to 16 (95%CI 0-37) per 10,000 births in populations with CMV seroprevalence of 30% to 95%(Figure 4C). In contrast, the number of children with sequelae attributable to primary infections ranged from 3 (95%CI 1-5) to 2 (95%CI 1-4) per 10,000 births in populations with CMV seroprevalence of 30% to 95%(Figure 4D).

The proportion of congenital infections with sequelae attributable to non-primary infections ranged from 29% (95%CI 2-70%) to 89% (95%CI 26-97%) in populations with CMV seroprevalence of 30% to 95% (Figure 5B). Non-primary CMV infections were estimated to account for the majority of children with CMV-related sequelae among populations with seroprevalence values of 63% and higher.

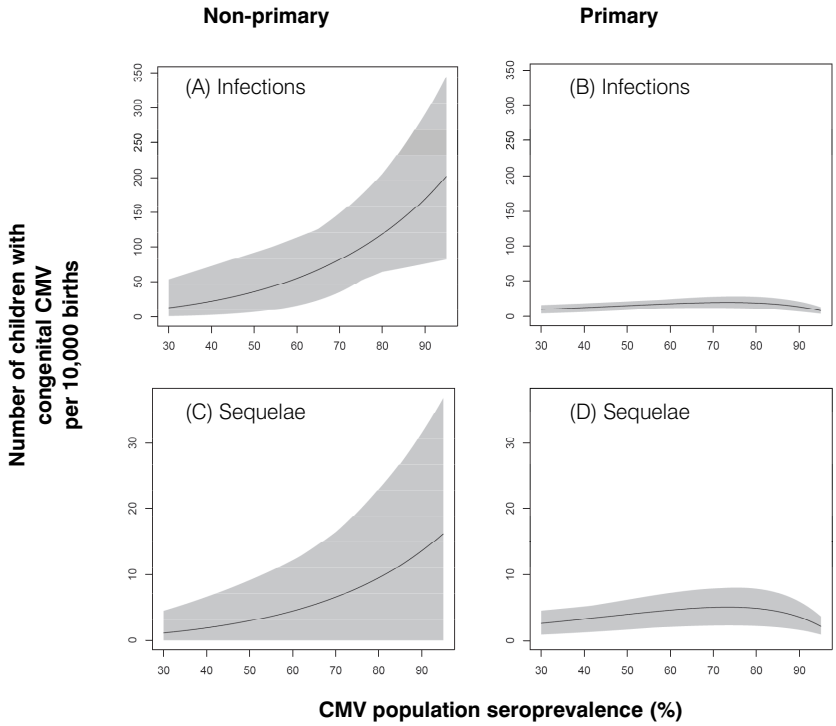

Figure 4 A/B: The estimated number of newborns with congenital CMV per 10,000 births, as a function of the CMV seroprevalence in the underlying population, classified by non-primary (A) and primary maternal infection (B).
C/D: The estimated number of children with congenital CMV-related sequelae per 10.000 births, as a function of the CMV seroprevalence in the underlying population, classified by non-primary (C) and primary maternal infection (D). Sequelae include sensorineural hearing loss, IQ≤70, chorioretinitis, microcephaly, seizures, paresis and paralysis, and death.[2] Grey zones represent 95%CIs.

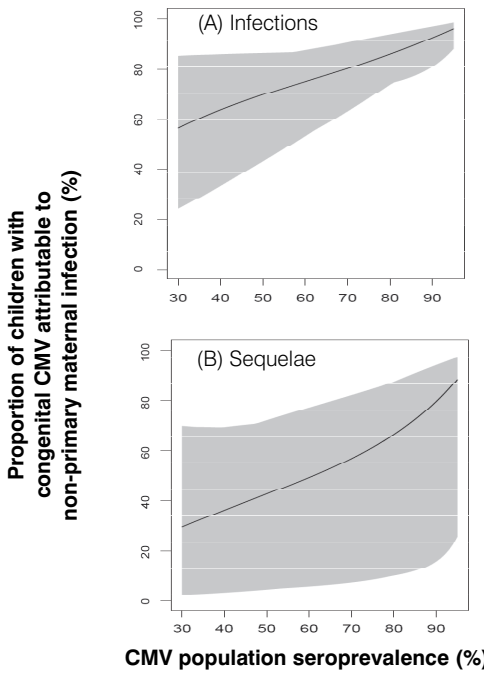

Figure 5 Estimated proportion (%) of children with congenital CMV (A) and CMV-related sequelae (B) born to seropositive mothers, relative to the total number of children with congenital CMV and CMV-related sequelae respectively, as a function of the seroprevalence in the underlying population.

Relative risk for seropositive women

The estimated risk for preconceptionally seropositive women of having a newborn with congenital CMV and CMV-related sequelae, compared to this risk for seronegative pregnant women (relative risk, RR), is shown in Figure 6. E.g., in a population with 50% seroprevalence, the relative risk for seropositive women of having newborn with congenital CMV was 2.3 (absolute risk for seropositive women / absolute risk for seronegative women in that population, 0.72% / 0.31%).

For all CMV seroprevalence values, seropositive women were at higher risk of having a congenitally-infected newborn than seronegative women (RR>1), with a relative risk of 3.0 (95%CI 0.7-27) to 1.3 (95%CI 0.6-3.5) in populations with CMV seroprevalence of 30% to 95% (absolute risks of 0.44% / 0.15% to absolute risks of 2.12% / 1.67%). In contrast, the risk of having a child with sequelae related to congenital CMV was lower for seropositive women (RR<1), for all seroprevalence values. This relative risk ranged from 0.97 (95%CI 0.06-12) to 0.41 (95%CI 0.05-1.80) in populations with CMV seroprevalence of 30% to 95% (absolute risk of 0.04% / 0.04% to absolute risks of 0.17% / 0.42%). This is similar to a 1.0 to 2.5 times higher relative risk for seronegative women compared to seropositive women.

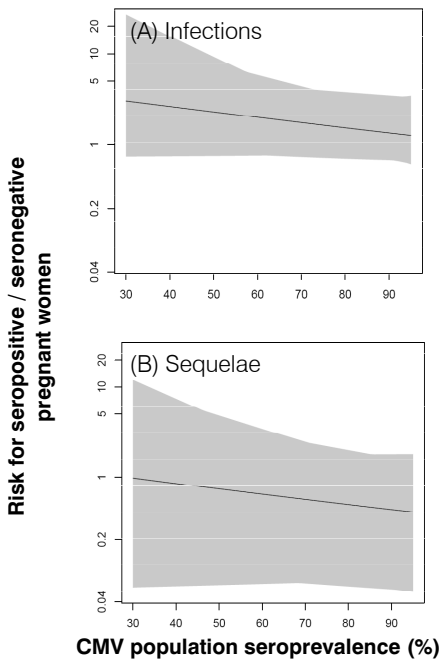

Figure 6 The estimated risk (RR) for preconceptionally seropositive pregnant women of having a child with congenital CMV (dotted line) and CMV-related sequelae (continuous line), relative to this risk for seronegative pregnant women, as a function of the seroprevalence.

Discussion

Using our model, we found that both the number and the proportion of newborns with congenital CMV infection attributable to non-primary maternal infections increased with CMV seroprevalence in the underlying population. Importantly, both the number and proportion of newborns with sequelae attributable to non-primary maternal infections was also highest in highly seroprevalent populations. Furthermore, seroimmune pregnant women were found to be at higher risk of having a congenitally infected newborn than seronegative pregnant women, for all population CMV seroprevalence values. This relative risk was up to three times higher among seroimmune pregnant women in populations with low CMV seroprevalence values, and decreased with CMV seroprevalence in the population. In contrast, seropositive pregnant women were at lower risk of having a newborn with sequelae than seronegative pregnant women.

Our findings are supported by earlier findings[7] and recent calculations on the absolute number of congenital CMV infections in the United States attributable to non-primary maternal infections.[5] Additional to these reports, our model predicted the contribution of non-primary infections for a wide range of CMV seroprevalence, and took into account an exponential effect of CMV seroprevalence on both the maternal-to-fetal transmission rate and the seroconversion rate. Furthermore, we added the risk for sequelae to these population-based estimates.

Parameters used in our model were based on data from previous studies listed in recent and extensive meta-analysis, and robustness of the parameters was assessed in our sensitivity analysis. It must be noted that the estimated proportion of newborns had wide 95%CIs, resulting from the denominator (total congenital infections), combined with the crude estimate of the risk for sequelae, and should therefore be interpreted with care.

It would be of interest to quantify the contribution of non-primary CMV infection on different sequelae seperately, since it may well be that sequelae associated with primary infection are more severe than sequelae associated with non-primary infection.[2] However, outcomes from studies assessing e.g. hearing impairment following non-primary maternal infections vary widely[2;4;35], and render it difficult to produce reliable estimates on the risk on these different sequelae to date. A complicating factor might be be that the severity of disease, including hearing loss, following primary infections may vary with gestational age at infection.[36;37] More detailed studies are needed to calculate the exact impact of non-primary maternal infections on the different sequelae seperately, as a function of the seroprevalence.

The apparent contradiction of maternal immunity as a risk factor for congenital CMV can be understood as follows. Once infected, previously seronegative pregnant women are at much higher risk of transmitting CMV to their fetuses compared to preconceptionally seropositive pregnant women. However, it is also necessary to include the risk of actually acquiring an infection. If this risk is also taken into account, seropositive pregnant women are at higher risk of having a congenitally infected newborn compared to preconceptionally seronegative pregnant women. The risk of re-infection or reactivation in seropositive pregnant women outweighs the combined risks of the risk of acquisition and transmission in seronegative pregnant women. Recent serological studies assessing strain-specific CMV antibody responses have shown that maternal re-infection with a new strain is a major source of congenital infection in seroimmune women, with re-infection occurring in 8% of the seroimmune pregnancies.[38] The circulation of CMV or the force of infection appeared to be highest in highly seroprevalent populations, based on age-specific seroprevalence data.[33;39] Differences in acquisition rates between high and low seroprevalent (sub)populations seem plausible given their difference in first exposure, and are likely based on environmental and behavioral differences.

Our data stress the relevance of non-primary maternal congenital CMV infection for the disease burden of congenital CMV, among all (sub)populations. Awareness of the risk for seroimmune pregnant women of having a congenitally infected and neurologically disabled newborn will have significant consequences for preventive strategies to reduce the disease burden of congenital CMV. Preventive measures such as hygienic behavior should be advised for both seronegative and seroimmune pregnant women. In that case, prenatal maternal serological screening will be futile as long as no adequate intervention is available. Awareness of the fact that CMV seroimmunity is only partially protective for congenital infection raises questions on the role of re-infections with new strains and reactivations of latent virus in seroimmune pregnant women. Passive and active immunization efforts will be challenged since the induction of a specific CMV immune response may not fully protect against fetal infection and disease, and an immunological correlate of full protection is lacking. A CMV glycoprotein B vaccine boosted immunity in CMV seropositive women [40], however a potential effect on maternal to fetal transmission rate and congenital CMV disease remains to be tested. In short, awareness of the paradox of maternal seroimmunity as a risk factor for congenital infection as addressed in this study will have significant consequences for preventive strategies including hygiene counseling, maternal serological screening, and immunization studies.

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Chapter 5

Awareness of congenital cytomegalovirus among doctors in the Netherlands

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Abstract

Background

Because of limited treatment options for congenital cytomegalovirus (CMV) infection, preventive strategies are important. Knowledge and awareness are essential for the success of preventive strategies.

Objectives

To investigate the knowledge of congenital CMV among doctors involved in mother and child care in the Netherlands.

Study design

A questionnaire on CMV infection was sent to doctors by snowball sampling. Knowledge concerning epidemiology, transmission, symptoms and signs of CMV infection in adults and children, and treatment options were evaluated.

Results

The questionnaire was completed by 246 doctors involved in mother and child care. The respondents estimated a prevalence of congenital CMV varying between 0.1 and 500 per 1000 live-born infants. The mean knowledge scores regarding transmission and postnatal symptoms increased with a more advanced career stage (i.e. older age). Gender and parenthood did not contribute to knowledge, but the field of expertise did. Respondents in the field of pediatrics had the highest mean score on postnatal symptoms and long-term effects. Respondents working in the field of gynecology and obstetrics were unaware of the precise transmission route of CMV. More than one-third of the respondents assumed that treatment was readily available for congenital CMV infection.

Conclusions

The knowledge of CMV infection among doctors in the Netherlands contained several gaps. Increasing knowledge and awareness is expected to enhance the prevention of transmission, to improve recognition, and to stimulate diagnostic investigations and follow-up programs.

Background

Congenital cytomegalovirus (CMV) infection is the most common congenital viral infection in newborns, with an estimated worldwide prevalence in live newborns of 0.64%.[1] Symptoms and signs are present at birth in 10–15% of these children, and another 15–20% will have sequelae that become apparent later in life.[2] We estimate that each year in the Netherlands approximately 800 children congenitally infected with CMV are born, of whom an estimated 160 will have long-term effects.[3] The symptoms and signs of congenital CMV, such as intrauterine growth retardation (IUGR), elevated liver enzymes, convulsions, and long-term effects such as developmental delay and permanent childhood hearing impairment[2] have a great impact on the lives of children and their parents. Although several vaccines are being developed and tested, no vaccine has been licensed for use. Furthermore, in most countries no prenatal or neonatal screening program for congenital CMV infection exists, and only limited data exist on the effectiveness of prenatal or postnatal treatments.

While awaiting treatment options, the burden of disease can be decreased by preventive strategies that reduce the risk of transmission of CMV to the pregnant woman.[4,5] A recent review of the implementation of educational hygiene interventions provides preliminary support for the positive effect of preventive strategies.[6] The success of preventive strategies depends on the active involvement of the doctors involved in mother and child care.[4] Awareness of the epidemiology, transmission, diagnosis and prevention of congenital CMV is essential for every doctor. Recent studies report on the knowledge of women of childbearing age, and obstetricians, concerning congenital CMV.[7,8] One-fifth of the women of childbearing age had heard of congenital CMV, but only very few had specific knowledge about the clinical symptoms and signs, or the modes of transmission or prevention, even when these women were medical professionals themselves.

Half of the women who were aware of congenital CMV had heard about the virus from a doctor.[7] A recent survey reported that most obstetricians do not include congenital CMV in their advisory consultation with their pregnant patients.[8] Increased awareness of congenital CMV is important, not only to reduce the transmission rate, but also to improve the recognition of symptoms and signs in congenitally infected children.[9] Such awareness could improve the subsequent diagnostic investigation and follow-up of these children, and allow treatment studies to be conducted.

Objectives

The objective of this study was to determine the knowledge of doctors involved in mother and child care in the Netherlands concerning congenital CMV.

Methods

Questionnaire development

A 12-item questionnaire on CMV, taking less than 4 min to complete, was developed. The questionnaire tested knowledge of the prevalence of congenital CMV; the symptoms and signs of CMV in healthy adults, newborns and children; the mode of transmission; and the treatment options. All but two questions regarding epidemiology and possible treatment were multiple choice. The possible answers were based on the literature, and included 20% false answers (i.e. non-symptoms). Multiple answers were accepted. Several demographic variables were asked for, including gender, age, parenthood and professional field. When a respondent had not heard of CMV at all, the questionnaire ended after recording the characteristics of the respondent. The questionnaire was pilot tested using a convenience sample, and ambiguous questions were rephrased. The questionnaire summary is given in Fig. 1.

What is the transmission route of cytomegalovirus?		
Air	Kissing	Breastfeeding
Sexual intercourse	Changing diapers	Don't know
Direct skin contact	Blood contact	
What is the most frequent presentation of CMV infection in immune competent adults?		
No symptoms	Not feeling well	Visual problems
Fever	Thrombosis	Don't know
Cardiac problems	Elevated liver enzymes	
What symptoms can be seen in newborns with congenital CMV infection?		
No symptoms	Microcephaly	Seizures
Petechiae	Growth retardation	Anal atresia
Elevated liver enzymes	Renal problems	Hearing loss
Congenital heart defect	Macrosomia	
What long-term effects can present in children with congenital CMV infection?		
Hearing loss	Visual problems	Obesity
Cognitive delay	Autism	Increased risk of malignancy
Cardiac problems	Seizures	Motor delay

Figure 1 Summary of CMV survey, including possible answers.

Sampling frame and questionnaire administration

The digital questionnaire, accompanied by a covering letter, was sent to the medical contacts of the researchers. Snowball sampling was used; all participants were asked to forward the link of the online questionnaire to medical colleagues.[10] These contacts were interns, residents, hospital-based senior doctors, general practitioners and medical researchers. In addition to the digital questionnaire, a hard-copy version was completed by attendees at two local specialist meetings (Department of Pediatrics and Department of Otolaryngology). The questionnaire was anonymous, and the response period was closed after 2 months. One month after closure, an information brochure on congenital CMV was sent to all respondents who had indicated that they were interested. In this study the analysis was restricted to the replies of the doctors directly involved in mother and child care.

Data analysis

All statistical tests were carried out using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA), with the significance level set at $P < 0.05$. Frequency responses to all questionnaire items were determined, and overall scores were calculated per questionnaire item. This overall score was based on the sum of the correctly stated true answers and the correctly not chosen false answers, assigning one point per correct answer. The maximum achievable score varied between 7 and 12 points, depending on the questionnaire item. Comparisons between different groups of respondents were made using χ^2 tests.

Results

The questionnaire was completed by 415 respondents, of whom 246 were involved in the care of mothers and children. The characteristics of the respondents and their mean scores on the questionnaire's topics are shown in Table 1. There were no significant gender differences in the mean scores. The mean knowledge scores regarding transmission and postnatal symptoms increased with a more advanced career stage (corresponding with an older age). Parenthood or plans to have children did not contribute significantly to knowledge of CMV infection. The field of expertise did contribute significantly to knowledge of CMV. Respondents in the field of infectious diseases had the highest mean knowledge score on transmission routes. The lowest (total) scores were achieved by general practitioners and otorhinolaryngologists. Respondents in the field of pediatrics had the highest mean score on postnatal

symptoms, as well as on knowledge of the long-term effects. The details and background variables of non-respondents could not be identified because of the sampling method used. The respondents estimated a prevalence of congenital CMV varying between 0.1 and 500 per 1000 live-born infants.

In Table 2, the number and proportion of stated true and false answers to CMV questionnaire items are shown. The relevant knowledge of respondents in pediatrics (postnatal symptoms and long-term effects) and respondents in gynecology and obstetrics (transmission route) is shown in detail. Fifty-six percent of the respondents were aware that CMV infection may not produce any symptoms in healthy adults. More than 50% of the respondents realized that microcephaly, growth restriction and hearing loss can be signs of congenital CMV in newborns, and the potential long-term effects were known by more than 65%. Half of the respondents thought air conduction was a true transmission route of congenital CMV. One-third of the pediatricians were aware that congenital CMV infection might not give rise to any symptoms at birth. Hearing loss and cognitive delay were accurately acknowledged as long-term effects of congenital CMV. Most of the respondents working in the field of gynecology and obstetrics were unaware of the precise transmission route of CMV. Of the respondents working in pediatrics, 55% were convinced that treatment options for congenital CMV infection were readily available, compared with 34.6% of the total respondents.

Table 1 Demographic variables of the respondents involved in mother and child care.

Characteristics	Number (%)	Mean transmission route score (max. possible = 7)	Mean adult symptom score (max. possible = 7)	Mean postnatal symptom score (max. possible = 12)	Mean long-term effect score (max. possible = 9)
Gender					
Male	65 (26.4)	3.2	4.9	7.4[a]	5.2
Female	181 (73.6)	3.5	4.8	7.6	5.2
Career stage					
Resident	181 (73.6)	3.3[a]	4.8	7.3[a]	5.0
Senior doctor	65 (26.4)	3.8	4.9	8.3	5.5
Parenthood					
Having children or pregnant	205 (83.3)	3.8	4.9	7.9	5.3
No plans for children	41 (16.7)	3.4	4.8	7.5	5.3
Field of expertise					
Pediatrics	85 (34.5)	4.2[a]	4.8[a]	8.9[a]	6.2[a]
Gynecology and obstetrics	18 (7.3)	3.2	4.9	8.2	5.4
Oto-rhinolaryngology	13 (5.3)	3.2	4.5	6.9	5.3
General practice	121 (49.2)	2.8	4.9	8.2	5.4
Infectious diseases (incl. medical microbiology)	9 (3.7)	5.1	5.1	8.2	5.7
Total	246	3.4	4.8	7.5	5.2

[a] $P < 0.05$.

Table 2 The number and percentage of stated "yes" responses per multiple-choice item on the CMV questionnaire for all respondents and for respondents in pediatrics and obstetrics and gynecology in more detail.

Knowledge concerning	Total number of respondents (n = 246) (%)	Number of pediatricians (n = 85) (%)	Number of obstetricians and gynecologists (n = 18) (%)
Transmission route			
True answers			
Kissing	129 (52.4)	40 (47.1)	7 (38.9)
Changing diapers	56 (22.8)	34 (40)	4 (22.2)
Breast milk	85 (34.6)	42 (49.4)	8 (44.4)
Blood contact	141 (57.3)	69 (81.2)	11 (61.1)
Sexual intercourse	98 (39.8)	43 (50.6)	8 (44.4)
False answers			
Air conduction	126 (51.2)	31 (36.5)	9 (50.0)
Direct skin contact	38 (15.5)	9 (10.6)	6 (33.3)
Symptoms in immune competent adults			
True answers			
No symptoms	137 (55.7)	60 (70.6)	10 (55.6)
Not feeling well	159 (64.6)	48 (56.5)	14 (77.8)
Fever	88 (35.8)	25 (29.4)	8 (44.4)
Elevated liver enzymes	72 (29.3)	21 (24.7)	5 (27.8)
False answers			
Cardiac problems	0 (0)	0 (0)	0 (0)
Thrombosis	0 (0)	0 (0)	0 (0)
Visual problems	5 (2.0)	1 (1.2)	2 (11.1)
Postnatal symptoms			
True answers			
No symptoms	50 (20.3)	27 (31.8)	3 (16.7)
Petechiae	71 (28.9)	45 (52.9)	5 (27.8)
Elevated liver enzymes	109 (44.3)	57 (67.1)	5 (27.8)
Microcephaly	138 (56.1)	73 (85.0)	12 (66.7)
IUGR	146 (59.3)	61 (71.8)	16 (88.9)
Hearing loss	138 (56.1)	67 (78.8)	12 (66.7)
Seizures	68 (27.8)	40 (47.1)	6 (33.3)
False answers			
Heart defect	53 (21.6)	20 (23.5)	1 (5.6)
Macrosomia	2 (0.8)	0 (0)	0 (0)
Renal problems	29 (11.8)	13 (15.3)	1 (5.6)
Anal atresia	1 (0.4)	0 (0)	0 (0)

Long-term effects			
True answers			
Hearing loss	161 (65.5)	78 (91.8)	13 (72.2)
Cognitive delay	171 (69.5)	77 (90.6)	15 (83.3)
Motor delay	89 (36.2)	42 (49.4)	6 (33.3)
Seizures	49 (19.9)	32 (37.6)	0 (0)
Autism	10 (4.1)	6 (7.1)	0 (0)
Visual problems	99 (40.2)	50 (58.8)	11 (61.1)
False answers			
Cardiac problems	39 (15.9)	14 (16.5)	1 (5.6)
Obesity	1 (0.4)	0 (0)	0 (0)
Increased risk for malignancy	4 (1.6)	2 (2.4)	0 (0)
There is treatment available	85 (34.6)	47 (55.3)	5 (27.8)

Note that multiple answers were accepted so the percentage adds up to more than 100%.

Discussion and conclusion

We investigated the knowledge of congenital CMV infection among doctors in the Netherlands involved in mother and child care. Several interesting findings were shown. First, doctors seemed to miscalculate the chance of encountering a child with congenital CMV infection in medical practice. There seemed to be a risk of underdiagnosis, since the prevalence of congenital CMV, internationally estimated to be 0.64%, was sometimes overestimated, but more frequently underestimated by respondents in this study. Secondly, preventive strategies are assumed to be effective only when doctors are sufficiently well informed to advise their patients properly. The data in this study show that most of the doctors were aware that most healthy adults and pregnant women do not experience any symptoms of a CMV infection. Worryingly, however, only one-fifth of the respondents, including those working with pregnant patients, were aware that kissing, and changing diapers, are risk factors for the transmission of CMV. Thirdly, including congenital CMV infection in the differential diagnosis in symptomatic newborns is crucially important. Since only half of the total respondents in this study and two-thirds of those working in pediatrics were aware that microcephaly, IUGR and hearing loss could be symptoms of congenital CMV, it seems possible that these children may be left undiagnosed, with possibilities for treatment and follow-up not explored. It is worrying that only 20% of all respondents and 32% of respondents in pediatrics realized that congenital CMV frequently does not give rise to any symptoms and signs at birth, and that 14% of these asymptomatic newborns will develop long-term sequelae.[2] Finally, we were surprised that 55% of the

respondents in the field of pediatrics thought that the antiviral therapy of newborns with congenital CMV infections is common practice in the Netherlands. Even though antiviral therapy has been shown to prevent hearing deterioration in newborns with symptomatic CMV infections, this practice is not yet widespread here.[11]

This study has several shortcomings: the sampling frame and questionnaire administration might have introduced bias, since the invitation started among the medical contacts of the researchers. These contacts might have had an advantage in knowing more about congenital CMV. Additionally, completing the questionnaire might have been subject to response bias, since respondents who felt unsure about their knowledge of CMV could refuse to respond to the questionnaire. This bias, however, might imply that the true knowledge concerning congenital CMV infection is even poorer than reported.

Medical information is available from various sources: Internet, TV, radio, newspapers and magazines. However, information is generally not sought if the public is not aware of a health risk or health problem. It is the responsibility of a doctor to be aware of the risks to a population in special situations, and supply information to reduce the health risk. When the doctor lacks awareness, this information is not available to the population or the individual patient. In the case of CMV it is especially important that doctors involved in the care of women who are or who may become pregnant are able to advise on the risk of congenital CMV and how this risk may be reduced. To date, information on congenital CMV is not regularly included in preconception and antenatal consultations. Fig. 2 gives a brief overview of what doctors should know about congenital CMV infection, for those working in pediatrics or obstetrics and gynecology.

In summary, this study focused on one of the essential aspects of successful preventive strategies for congenital CMV infections: the knowledge of doctors involved in mother and child care. Consistent with earlier findings, we show that most doctors concerned with mother and child care in the Netherlands do not possess optimal knowledge on CMV. The results of this study can be used for discussions on awareness and relevant knowledge for each specific medical field, individualized education for doctors, and for the development of preventive strategies. Awareness and knowledge will subsequently improve the recognition of early and late symptoms and signs, improve diagnostic and follow-up programs, and might even promote the development of evidence-based treatment in the near future.

Pediatricians	Obstetricians and gynecologists
Presentation of congenital infection in newborns • Asymptomatic • Growth retardation and/or microcephaly • Hearing loss • Thrombocytopenia • Elevated liver enzymes	Presentation of CMV infection in adults • Asymptomatic • Fever • Elevated liver enzymes
Late effects of congenital infection • Hearing loss • Developmental delay	Transmission route • Body fluids • Blood contact
Estimated prevalence in newborns 0.64% worldwide	Preventive strategies • Wash hands after changing diapers • Aviod kissing young children on the mouth • Don't share food, drink or cutlery with young children

Figure 2 What doctors should know about congenital CMV infection.

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Chapter 6

Evaluation of DNA extraction methods for dried blood spots in the diagnosis of congenital cytomegalovirus infection

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Abstract

Background

Dried blood spots (DBS) may be valuable in the diagnosis of congenital cytomegalovirus (CMV) infection. However, the 2007 European Quality Control for Molecular Diagnostics (QCMD) proficiency testing programme showed that CMV DNA detection in DBS was lacking sensitivity in a considerable number of participating laboratories.

Objective

To compare DNA extraction methods for DBS for detecting CMV. Sensitivity and applicability of the methods for high-throughput usage were assessed.

Study design

Guthrie cards were spotted with CMV DNA-positive whole blood (n = 15). DNA was extracted from the DBS using different extraction methods, followed by CMV amplification by means of real-time PCR.

Results

Significant differences between the extraction methods with respect to the sensitivity were found. Optimal sensitivity was achieved when samples were tested in triplicate, demonstrating that the methods in general operated around their detection limits. Triplicate testing using the protocol by Barbi et al. [Barbi M, et al. Cytomegalovirus DNA detection in Guthrie cards: a powerful tool for diagnosing congenital infection. *J Clin Virol* 2000;17:159–65], representing the most sensitive methods, resulted in sensitivities of 100%, 86%, and 50% for DBS with CMV DNA loads of 5–4, 4–3, and 3–2 $\log_{10}$ copies/ml, respectively. This indicates that sensitivity limitations apply in the clinically relevant concentration range. Few methods appeared suitable for 96-well format high-throughput testing.

Discussion

When considering universal neonatal screening for congenital CMV infection, an assay which is both sensitive and applicable for high-throughput testing is required. The protocol by Barbi et al. and the BioRobot Universal System appear appropriate candidates currently available for 96-well format application in neonatal screening using DBS.

Introduction

Cytomegalovirus (CMV) infection is the most common cause of congenital infection worldwide with an overall birth prevalence of 6–7 per 1000 births.[1,2] About 12% of the live-born infants with congenital CMV infection are symptomatic at birth.[1,2] Of the children asymptomatic at birth, an additional 11–13.5% will develop permanent sequelae in the following years.[1–3] The most frequently encountered symptom of congenital CMV infection is sensorineural hearing loss (SNHL). Congenital CMV infection is responsible for 15–20% of SNHL in infants and children.[4,5]

Neonatal blood collected on filter paper within the first week of life (dried blood spots, DBS) has been proven useful for (retrospectively) diagnosing congenital cytomegalovirus (CMV) infection. The sensitivity of CMV DNA detection in DBS reported in literature is 71–100%, depending on the method used and the population tested.[6–11] However, the 2007 Quality Control for Molecular Diagnostics (QCMD) proficiency testing programme in which 33 European and South African laboratories participated, showed that CMV DNA detection in DBS was lacking sensitivity in a considerable number of participants. Only 50% of the laboratories were able to detect CMV DNA in a DBS sample with a load of 9.4×10^3 (4.0 $\log_{10}$) copies/ml whole blood.[12]

Currently, several non-commercial and commercial DNA extraction methods for DBS are available. A number of reports evaluating DNA extraction methods for DBS have been published. However, comparison of these data is complicated by inter-study differences, such as the origin of the samples, and the input and output volumes.[6–11]

The aim of our study was to test a panel of DNA extraction methods for DBS currently available. CMV-positive whole blood samples from transplant patients were spotted and DNA was extracted using the various methods, with identical input and output volumes, followed by CMV DNA amplification by real-time PCR. Sensitivity and applicability of the methods for high-throughput usage were determined.

Methods

Dried blood spots (DBS)

DBS samples were prepared by spotting CMV-positive EDTA-anticoagulated whole blood from transplant recipients with a broad range of CMV DNA loads (range 2–5 $\log_{10}$ copies/ml whole blood, $n = 15$) on Whatman 903 filter paper (kindly provided by Bert Elvers, RIVM, The Netherlands). The samples were air-dried, stored at room

temperature and tested within 3 months after spotting. In addition, CMV DNA-negative EDTA-anticoagulated whole blood from CMV-seronegative healty volunteers was spotted and used as negative controls. Furthermore, DBS from the QCMD CMV DBS 2007 panel (manufactured by Sandro Binda and Maria Barbi, Dept. of Public Health-Microbiology-Virology, University of Milan, Italy) were used to further analyse the protocol previously published by Barbi et al.,[6] representing the most sensitive methods.

Extraction of DNA from whole blood

CMV loads of the EDTA-anticoagulated whole blood from transplant recipients were determined prior to spotting using 200 µl for DNA extraction with the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Almere, The Netherlands). DNA extraction was followed by CMV amplification (see Section 2.12).

Extraction of DNA from DBS

DNA was extracted from DBS using the following extraction methods: the protocol described by Barbi et al.,[6] the QIAamp DNA Investigator Kit (QIAGEN), the BioRobot Universal System (QIAGEN), the MagNA Pure LC (Roche Diagnostics), the NucliSens easyMAG (bioMeriéux), the QIAsymphony (QIAGEN), and Dynabeads Silane (Invitrogen). Sample input per tube/well was 3 punches, each measuring 3.2 mm in diameter, corresponding with approximately 9 µl dried blood per tube/well for all extraction methods tested. DBS were punched using an automated plate punch type 1296-071 (Perkin Elmer-Wallac, Zaventem, Belgium). For all extraction methods, samples were tested in triplicate with a negative control punch between each sample. Output volume was 100 µl for all extraction methods tested. DNA extraction was followed by CMV amplification (see Section 2.12).

Since the above-mentioned fixed input and output volumes meant a significant deviation from the original protocol by Barbi et al. (dictating 1 punch input and 35 µl output volume), the original protocol by Barbi et al. (unmodified) was tested as well.

Extraction of DNA from DBS using the protocol by Barbi et al. (unmodified)

DNA was extracted from DBS using the protocol described by Barbi et al.[6] (details obtained by personal communication). One punch of 3.2 mm per tube (in triplicate) was incubated at 4 °C overnight in 35 µl Minimum Essential Medium (+Earle's, +25 mM HEPES, −l-glutamine, Gibco/Life Technologies, Breda, The Netherlands) without additives in 96-well cluster tube strips. An aliquot of phocine herpes virus (PhHV) was

added as nucleic acid isolation and PCR inhibition control, as described previously.[13] Incubation was followed by heating at 55 °C for 60 min, and 100 °C for 7 min in a thermal cycler. After rapid cooling at 4 °C, the sample was centrifuged at 3220 × g for 15 min. The supernatant was transferred to a 96-well plate, frozen at −80 °C for at least 1 h, and thawed. This protocol resulted in an output solution which was approximately 20% more concentrated than when using the modified protocol by Barbi et al. described below.

Extraction of DNA from DBS using the protocol by Barbi et al. (modified)
Essentially the same procedure was followed for the modified protocol by Barbi et al., except that 3 punches of 3.2 mm per tube (in triplicate) were incubated in 125 µl Minimum Essential Medium, obtaining an output volume of 100 µl.

Extraction of DNA from DBS using the QIAamp DNA Investigator Kit
DNA was extracted using the QIAamp DNA Investigator Kit (column-based manual extraction) following the protocol "Isolation of total DNA from FTA and Guthrie cards" with a modification in the elution buffer according to the manufacturer's recommendations. Briefly, 280 µl buffer ATL and 20 µl proteinase K were added to the punches in screw-capped tubes, followed by vortexing, and incubation at 56 °C while shaking at 900 rpm for 1 h. After addition of 300 µl buffer AL (with 1 µg carrier RNA and internal PhHV control), the mix was pulse-vortexed and incubated at 70 °C while shaking at 900 rpm for 10 min. Additionally, 150 µl ethanol (96–100%) was added, the sample was pulse-vortexed, and the mix was transferred to the QIAamp MinElute column and centrifuged. The column was washed with 500 µl buffer AW1, 700 µl buffer AW2, and 700 µl ethanol (96–100%) subsequently, followed by drying of the column membrane at room temperature for 10 min, and eluting of DNA with 100 µl buffer AE (provided with the QIAamp DNA Mini Kit).

Extraction of DNA from DBS using the BioRobot Universal System
DNA extraction using the BioRobot Universal System (columnbased automated extraction) was performed using the QIAamp Investigator BioRobot Kit with manual pretreatment according to the manufacturer's recommendations. Tests were performed by QIAGEN application specialists in application laboratory Hilden, Germany. Briefly, 280 µl buffer ATL (with 2.75 µg carrier RNA and internal PhHV control) and 20 µl proteinase K were added manually to the punches in a QIAGEN 96-well S-Block. Samples were incubated at 56 °C overnight while shaking at 900 rpm in

a heatable shaker (Eppendorf Thermomixer Comfort with Thermoblock for Microtiter and Deepwell Plates with lid). After pretreatment, the supernatant was transferred manually to an empty S-Block and loaded on the BioRobot Universal System running the protocol "QIAamp DNA BloodCard UNIV" with an input volume of 300 and 100 µl elution volume.

Extraction of DNA from DBS using the MagNA Pure LC

DNA extraction using the MagNA Pure LC (magnetic particlebased automated extraction) was performed with manual pretreatment according to manufacturer's recommendations (Ref. 14, with minor modifications). The MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche Diagnostics, Almere, The Netherlands) was used. Briefly, pretreatment was performed by adding a premix of 230 µl Bacteria Lysis/Binding Buffer, 20 µl proteinase K, and internal PhHV control to the punches in screw-capped tubes, vortexing and incubating at room temperature overnight. The following day, the mixture was incubated at 95 °C for 10 min, centrifuged briefly, and cooled at 4 °C. Supernatant was transferred manually to the MagNA Pure LC running the protocol "DNA Isolation Kit III" with an input volume of 200 µl and an elution volume of 100 µl.

Extraction of DNA from DBS using the QIAsymphony

DNA extraction using the QIAsymphony (magnetic particlebased automated extraction) was performed using the QIAsymphony DNA Mini Kit with manual pretreatment according to manufacturer's recommendations since this method was originally not designed for application of DBS (however currently in development). Pretreatment was performed following the QIAsymphony protocol "Pretreatment of Tissues" with minor modifications. Briefly, 180 µl buffer ATL (with added internal PhHV control) and 20 µl proteinase K were added to the punches in screw-capped tubes, followed by incubation at 56 °C with shaking at 900 rpm overnight. Supernatant was loaded manually on the QIAsymphony (magnetic particles based) running the protocol "Purification of DNA from tissues, cultured cells and bacterial cultures/DNA Tissue Low Content" with an input volume of 200 and 100 µl elution volume.

Extraction of DNA from DBS using the easyMAG

DNA extraction using the easyMAG (magnetic particle-based automated extraction) was performed using the NucliSENS easy-MAG Extraction Kit with manual pretreatment according to manufacturer's recommendations (Ref. 9, with minor modifications).

Briefly, punches were transferred into 2 ml NucliSens easyMAG lysis buffer in 10 ml lidded glass tubes, and incubated by gently rocking on a roller in horizontal position at room temperature for 30 min. After spinning down potential filter fibers at 1500 × g for 15 s, supernatant was loaded on the easyMag manually, running the off board extraction protocol (Generic, version 2.0.1). Internal PhHV control was added to extraction buffer 3, the elution volume was 100 µl.

Extraction of DNA from DBS using Dynabeads Silane

Dynabeads Silane extraction (magnetic particle-based manual extraction) was performed using the Dynabeads Silane viral NA kit with pretreatment according to manufacturer's suggestions since this method was originally not designed for application of DBS (however currently in development). Briefly, 200 µl phosphate buffered saline was added to the punches in screw-capped tubes and incubated at 85 °C for 10 min, followed by incubation with 20 µl proteinase K (20 mg/ml, Invitrogen/ Life Technologies, Breda, The Netherlands) at 55 °C for 10 min. Additionally, the mixture was incubated with 300 µl viral NA lysis buffer (including internal PhHV control) on a rotating wheel at room temperature for 10 min. The supernatant was transferred to an empty tube and suspended in 150 µl isopropanol and 50 µl Dynabeads suspension (silica-like magnetic beads) and incubated on a rotating wheel at room temperature for 10 min. Using the magnet, supernatant was removed and the Dynabeads were washed twice with 850 µl Washing Buffer 1 and 450 µl Washing Buffer 2. After drying the bead-pellet at room temperature for 10 min, the pellet was resuspended in 100 µl viral NA elution buffer and incubated at 70 °C for 3 min. Using the magnet, beads were separated from the supernatant, which was harvested.

Quantitative real-time PCR

CMV DNA amplification was performed by means of an internally controlled quantitative real-time PCR as described previously[13] with minor modifications. Briefly, 10 µl of DNA extract was added to 40 µl PCR pre-mixture obtaining final concentrations of 0.5 µM forward CMV primer, 0.5 µM reverse CMV primer, 0.2 µM CMV TaqMan probe, 0.3 µM forward PhHV primer, 0.3 µM reverse PhHV primer, 0.05 µM PhHV TaqMan probe, 3 mM $MgCl_2$, and 25 µl HotStar Master mix (QIAGEN, Hilden, Germany). The PCR running 50 cycli was carried out in an iQ5 Multi-colour Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands), amplifying a 126-bp fragment from the CMV immediate-early antigen region. Quantification was performed using a dilution series of titrated CMV (Advanced Biotechnologies Inc., Columbia, MD, USA) as an external standard.

Qualitative and quantitative data analysis

Qualitative data of DBS (n = 15), tested in triplicate, were analysed as follows. DBS were counted positive when ≥1 of the triplicates tested positive.[7] Additionally, to compare single and triplicate testing, ordinal means of the triplicates were calculated and considered the result of single testing (thereby enhancing the distinctive character compared with true single testing). Statistical analysis of ordinal data was performed using the Wilcoxon signed ranks test (two-tailed). The sign test (two-tailed) was used for comparison of single and triplicate testing.

In the quantitative data analysis, undetected samples were assigned the minimum detected load and mean loads were calculated per spotted CMV load categories. CMV DNA loads detected in 3 punches of each 3.2 mm, corresponding with in total approximately 9 µl dried blood, were converted to CMV DNA loads per ml spotted whole blood.

High-throughput applicability

Throughput characteristics determined were the maximum number of tubes/wells per run and the applicability of an automated system.

Results

Qualitative results

Qualitative results of the extraction methods tested are shown in Fig. 1(A) and (B). Fig. 1(A) shows the number of detected CMV-positive DBS (%) per method, comparing single testing (left) with triplicate testing (the DBS was counted positive when ≥1 of the triplicates was positive,[7] right). Single testing of DBS resulted in CMV DNA detection ranging from 32% (4.8/15) using the extraction method Dynabeads Silane, to 73% (11.0/15) using the protocol by Barbi et al. (unmodified). The highest number of samples were detected using the protocol by Barbi et al. (unmodified), the QIAamp DNA Investigator Kit (71%, 10.7/15), the BioRobot Universal System (67%, 10.0/15), the modified protocol by Barbi et al. (67%, 10.0/15), and MagNA Pure LC (62%, 9.3/15), respectively. The protocol by Barbi et al. (unmodified) was significantly more sensitive than extraction using the QIAsymphony (54%, 8.2/15, P = 0.031, Wilcoxon signed ranks test), the easyMAG (53%, 8.0/15, P = 0.031) and Dynabeads Silane (P = 0.003). Extraction using Dynabeads Silane was significantly less sensitive than all other extraction methods tested (P ≤ 0.039). For all methods, sensitivity was enhanced when testing was performed in triplicate compared with single testing (P = 0.008, sign test).

Fig. 1(B) shows the number of detected CMV-positive DBS (%) per spotted CMV DNA load category resulting from single testing. DBS with low CMV DNA loads (2–3 $\log_{10}$ copies/ml whole blood, n = 2) were not detected by four out of eight methods in any of the triplicates. When testing DBS with moderate CMV loads (3–4 $\log_{10}$ copies/ml whole blood, n = 7), the number of detected samples varied from 17% (1.2/7) using the extraction method Dynabeads Silane, to 67% (4.7/7) using the protocol by Barbi et al. (unmodified). DBS with high CMV DNA loads (4–5 $\log_{10}$ copies/ml whole blood, n = 6) tested positive in all triplicates using the protocol by Barbi et al. (unmodified and modified), and the QIAamp DNA Investigator Kit.

Sensitivity of CMV DNA detection per CMV load category was increased when samples were tested in triplicate (not shown in graph). When tested in triplicate, all DBS with CMV DNA loads of 3–4 $\log_{10}$ copies/ml (100%, 7/7) were detected using the BioRobot Universal System. All DBS with CMV DNA loads of 4–5 $\log_{10}$ copies/ml (100%, 6/6) were detected by all methods tested, except for Dynabeads Silane. Triplicate testing using the protocol by Barbi et al. (unmodified) resulted in sensitivities of 50% (1/2), 86% (6/7), and 100% (6/6) for spotted CMV DNA loads of 2–3, 3–4, and 4–5 $\log_{10}$ copies/ml, respectively.

All 120 CMV DNA-negative control samples (15 per extraction method) tested negative. No PCR inhibition was found using any of the extraction methods.

Quantitative results

Quantitative results of the DNA extraction methods tested are shown in Fig. 1(C). Depicted are the detected mean CMV DNA loads of triplicates per spotted CMV DNA load categories. Detected CMV DNA loads in DBS with spotted CMV DNA loads of 2–3, and 3–4 $\log_{10}$ copies/ml were lower than the spotted load category in six out of eight and five out of eight methods tested, respectively. CMV DNA loads detected in DBS with high spotted CMV DNA loads (4–5 $\log_{10}$ copies/ml) were within the ranges of the spotted load category in seven out of eight methods tested.

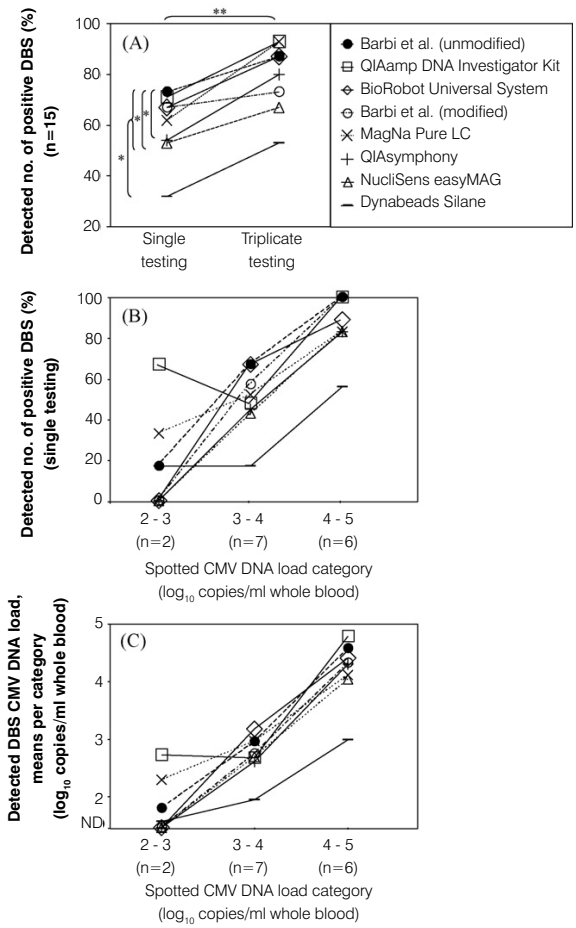

Figure 1 (A) Qualitative results of CMV DNA detection from DBS of the extraction methods tested, demonstrating the effect of triplicate testing on sensitivity. Left: single testing, right: triplicate testing (DBS was counted positive when ≥1 of the triplicates was positive). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.
(B) Qualitative results of CMV detection from DBS of the extraction methods, per spotted CMV DNA load category, after single testing.
(C) Quantitative results of CMV DNA detection from DBS of the extraction methods tested, per spotted CMV DNA load category. Depicted are detected mean CMV DNA loads of triplicates per category. DBS, dried blood spot; CMV, cytomegalovirus.

QCMD panel

The QCMD CMV DBS 2007 panel (manufactured by Sandro Binda and Maria Barbi, Dept. of Public Health-Microbiology-Virology, University of Milan, Italy) was used to test the extraction method by Barbi et al. (unmodified, tested and analysed in triplicate), representing the most sensitive methods. Results are shown in Table 1. DBS with spotted CMV DNA loads from 3.9×10^6 (6.6 $\log_{10}$)to9.4×10^3 (4.0 $\log_{10}$) copies/ml were detected in all triplicates. One out of two DBS with spotted CMV DNA loads of 7.3×10^2 (2.9 $\log_{10}$) copies/ml was detected (in 1/3 triplicates). Only 50% and 4% of the QCMD CMV DBS 2007 participants detected CMV DNA in DBS with spotted loads of 9.4×10^3 (4.0 $\log_{10}$) and 7.3×10^2 (2.9 $\log_{10}$) copies/ml, respectively.[12]

Table 1 Qualitative and quantitative results of CMV detection in the QCMD CMV DBS 2007 panel using the DNA extraction protocol by Barbi et al. (unmodified), and the qualitative results of all QCMD participants. Quoted with permission of QCMD. QCMD, Quality Control for Molecular Diagnostics; CMV, cytomegalovirus; DBS, dried blood spot.

QCMD CMV DBS 2007 panel[a]	Results using Barbi et al. (unmodified)		Results of all QCMD participants
Spotted viral load (copies/ml whole blood)	Qualitative results	Detected viral load (copies/ml whole blood)	% correct qualitative results
3.9×10^6	Positive (3/3 triplicates)	2.2×10^6	100
9.6×10^5	Positive (3/3)	3.0×10^5	96
8.8×10^4	Positive (3/3)	4.4×10^4	93
9.4×10^3	Positive (3/3)	5.0×10^3	52
9.4×10^3	Positive (3/3)	4.2×10^3	48
7.3×10^2	Positive (1/3)	1.1×10^2	7
7.3×10^2	Negative	–	0
Negative	Negative	–	96
Negative	Negative	–	96

[a] Panel manufactured by Sandro Binda and Maria Barbi, the Dept. of Public Health-Microbiology-Virology, University of Milan, Italy.

High-throughput applicability

Throughput characteristics of the DNA extraction methods tested are shown in Table 2. Methods applicable for 96-well format (32 samples/run when testing in triplicate) were the protocol by Barbi et al., the BioRobot Universal System, and the QIAsymphony. All automated systems tested required a manual pretreatment step (no primary tube input format for DBS was available).

Table 2 Throughput characteristics of the DNA extraction methods tested.

	No. of tubes/ wells per run	Manual/ automated	Input type	Throughput
Dynabeads Silane[a] (Invitrogen)	1–16	Manual	Tube	Low
QIAamp DNA Investigator Kit[b] (QIAGEN)	1–24	Manual	Tube	Low
NucliSens easyMAG[a] (bioMérieux)	1–24	Automated[c]	Tube	Medium
MagNA Pure LC[a] (Roche Diagnostics)	1–32	Automated[c]	Tube	Medium
Barbi et al. (un)modified	1–96	Manual	Tube or 96-well plate	Medium
QIAsymphony[a] (QIAGEN)	1–96	Automated[c]	Tube or 96-well plate	High
BioRobot Universal System[b] (QIAGEN)	8–96	Automated[c]	96-Well plate	High

[a] Magnetic particle-based extraction.
[b] Column-based extraction.
[c] Manual pretreatment step, no primary tube input format for DBS available.

Discussion

The data presented here show that sensitivity of CMV DNA detection in DBS varies widely depending on the DNA extraction method used. The most sensitive methods were the protocol described by Barbi et al. (unmodified and modified), the QIAamp DNA Investigator Kit, the BioRobot Universal System, and the MagNA Pure LC. Interestingly, the unmodified protocol by Barbi et al. using only 1 punch was not less sensitive than the modified protocol by Barbi et al. using 3 punches, probably resulting from a DNA concentration effect: the unmodified protocol by Barbi et al. resulted in an output solution which was approximately 20% more concentrated than the modified protocol. For all extraction methods, optimal sensitivity was achieved when samples were tested in triplicate. Triplicate testing using the protocol by Barbi et al. resulted in sensitivities of 100%, 86%, and 50% for DBS with CMV DNA loads of 5–4, 4–3, and 3–2 $\log_{10}$ copies/ml, respectively. DBS with low spotted CMV loads had lower detected loads reflecting the presence of not detected samples. The protocol by Barbi et al., the QIAsymphony, and the BioRobot Universal System were suitable for 96-well format testing, which would be a requirement for application in newborn screening laboratories. It must be stressed that in the automated systems tested, pretreatment had to be performed manually (lacking primary tube input for DBS), thereby significantly increasing hands-on time. Considering cost-efficacy, the protocol by Barbi et al. has the advantage of the lower costs (<0.30€ per sample, triplicate testing) compared to the other methods tested (7–15€ per sample, triplicate testing).

Several reports have been published comparing a limited amount of DNA extraction methods for DBS.[6–11] However, comparison of these data is complicated by inter-study differences. Potential variables influencing the sensitivity are the origin of the DBS sample (e.g. spiked virus versus clinical samples from symptomatic or asymptomatic patients with congenital CMV infection), the amount of dried blood volume used, the elution volume, and the amplification method. The QIAamp DNA Blood Mini Kit (QIAGEN) has been reported to have a 95% sensitivity at a spotted CMV DNA load of 3.6 $\log_{10}$ copies/ml in an experiment with diluted blood from a transplant recipient, using a whole DBS (50 µl dried blood).[10] A modified QIAamp DNA Micro Kit (QIAGEN) protocol has been described to have a sensitivity of 100% when testing DBS from seven neonates with congenital CMV (of whom three known to be symptomatic), using a whole DBS.[8] Soetens et al. reported 73% sensitivity of extraction by the NucliSens easyMAG when testing DBS from 53 asymptomatic and 2 symptomatic congenital infected neonates, using a whole DBS.[9] Considering DNA extraction by means of heat shock, Yamamoto et al. reported a 71.4% sensitivity of heat shock in combination with a nested PCR when testing DBS from seven congenitally infected children (of whom five symptomatic), using 3 $\times$ 6 mm punches.[11] The highest detection rate using heat shock was reported by Barbi et al., whose method had a 100% sensitivity when testing DBS from 72 congenital infected babies (of whom 26 symptomatic), using one 3 mm punch tested in triplicate followed by nested PCR.[6] In our study, the influence of potential differences was excluded by using identical clinical samples (samples from transplant recipients, containing both extra-and intracellular CMV DNA), identical input and output volumes, and an identical amplification assay for all extraction methods tested.

The sample size in our study was small, but partially amended by calculating ordinal means of triplicates, thereby enhancing the differences. However, the power of the study did not yet allow to detect potential other statistically significant differences between the extraction methods.

A number of studies have been published on the viral load levels in whole blood of neonates with congenitally infected CMV. Halwachs-Baumann et al. reported a median viral load of 2.3×10^3 (3.4 $\log_{10}$) copies/ml cord vein blood in 18 neonates with congenital CMV. No significant difference was found in virus load between children that were symptomatic (n = 7) or asymptomatic (n = 11) at birth.[15] In contrast, Boppana et al. reported a mean peripheral blood CMV DNA load of 4.0×10^5 (5.6 $\log_{10}$)copies/ml in congenitally infected symptomatic newborns (n = 18), which was significantly higher than the mean load of asymptomatic newborns:

8.2×10^4 (4.9 $\log_{10}$) copies/ml (n = 58). Among asymptomatic newborns, those with hearing loss at follow-up had a significantly higher mean CMV DNA load (8.7×10^5, 5.9 $\log_{10}$ copies/ml, n =4) than those with normal hearing (1.1×10^4, 4.0 $\log_{10}$ copies/ml, n = 54).[16] The results of Boppana et al. corresponded with data from Lanari et al. and Revello et al., both reporting a significantly higher mean CMV DNA load in symptomatic newborns (3.2 $\log_{10}$ copies/10^5 PMNLs and 3000 copies/10^5 PBL, respectively) than in asymptomatic newborns (2.8 $\log_{10}$ copies/10^5 PMNLs and 30 copies/10^5 PBL, respectively).[17,18] In our study, the 86% sensitivity of CMV DNA detection in DBS using the extraction protocol by Barbi et al. was 3–4 $\log_{10}$ copies/ml. This sensitivity combined with the median viral load of 3.4 $\log_{10}$ copies/ml mentioned by Halwachs-Baumann et al. would implicate that a significant amount of cases with congenital CMV would not be detected even using one of the most sensitive methods available. In contrast, when considering the mean viral loads of 4.0 and 5.9 $\log_{10}$ copies/ml in asymptomatic newborns with respectively normal hearing and hearing loss at follow-up mentioned by Boppana et al., the clinical significance of loads below the detection limit are debatable.

The usage of dried urine specimens on filter paper (placed in diapers) has been suggested by Nozawa et al. as urine generally contains higher CMV loads than blood.[19] Though not evaluated in our study, it is likely that the above described extraction methods will be applicable to dried urine specimens on filter paper as well. When considering universal neonatal screening for congenital CMV infection, a cost-efficient assay which is both sensitive and applicable for 96-well format testing, using only a very small amount of dried blood, is required. In our hands, the protocol by Barbi et al. and the BioRobot Universal System appear appropriate candidates currently available for application in neonatal screening. Further studies are needed to optimize test characteristics (e.g. primary tube input) and to assess the clinical relevance of the detection limit in the intended population of asymptomatic newborns at risk for developing hearing loss later in life.

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Chapter 7

Extraction of DNA from dried blood in the diagnosis of congenital CMV infection

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Summary

Viral DNA detection in dried blood spotted on filter paper, dried blood spots (DBS), is valuable in the diagnosis of viral infections, with at the moment congenital cytomegalovirus (CMV) being the most common application. CMV detection in clinical samples taken within the first 2-3 weeks after birth differentiates congenital CMV infection from the in general harmless postnatally acquired cytomegalovirus infection. DBS render the possibility to diagnose congenital CMV infection retrospectively, e.g. when late-onset hearing loss, the most frequently encountered symptom of congenital CMV infection, becomes manifest. Additionally, CMV DNA detection in DBS can be of usage in recently advocated newborn screening on congenital CMV infection. The procedure of CMV DNA detection in DBS consists of two separate steps: 1. DNA extraction from the DBS, followed by 2. CMV DNA amplification. Here, we describe two efficient methods for the extraction of DNA from DBS. Sensitivity, specificity, and applicability of the methods for high-throughput usage are discussed.

Introduction

Newborn blood taken within a few days after birth and dried on filter paper (dried blood spots, DBS), are widely used for newborn screening on metabolic diseases. Additionally, DBS have been proven valuable and are increasingly used in the diagnosis of viral infections. It is most often used in the diagnosis of congenital cytomegalovirus (CMV) infection, the most common congenital viral infection worldwide.[1] Congenital CMV infection can be retrospectively diagnosed using DBS, differentiating congenitally acquired CMV infection from postnatally acquired CMV infection, which is generally much less harmful. DBS render the possibility to diagnose congenital CMV infection when the most common symptom of congenital CMV infection, late-onset hearing loss, becomes manifest. Additionally, CMV detection in DBS can be of usage in recently advocated newborn screening on congenital CMV infection.[2-11]

CMV DNA detection in DBS includes DNA extraction followed by CMV DNA amplification and is increasingly used in clinical virological laboratories worldwide. Whereas detection of CMV DNA in blood and other clinical samples is a routine diagnostic procedure, the extraction of CMV DNA from filter paper is still challenging due to the limited amount of dried blood available; one whole spot of 1 cm in diameter equals approximately 50 µL blood, and one punch of 3 mm in diameter, frequently used for routine metabolic screening, contains as little as 3-5 µL blood. Thus, optimal DNA extraction is crucial in the procedure for CMV DNA detection in DBS.

Currently, several non-commercial and commercial DNA extraction methods for DBS are available. A number of reports evaluating extraction methods for DBS in the diagnosis of congenital CMV infections have been published.[3-5,12-25] Significant differences between extraction methods with respect to the analytical and clinical sensitivity are reported, ranging from 35% to 100%[3,14-18,20,21,24-26] depending on the extraction method used and the population tested. Optimizing DNA extraction protocols, PCRs, and algorithms, e.g. by means of performing independent triplicate testing, have been shown to increase analytical sensitivity significantly.[15,16,18] Triplicate testing (of one punch of 3 mm in diameter per tube) using the heat-shock protocol by Barbi et al[12], shown to be one of the most sensitive methods[15], results in analytical sensitivities of approximately 100%, 86% and 50% for DBS with CMV DNA loads of 5-4, 4-3, and 3-2 $\log_{10}$ copies/ml, respectively.[15] This indicates that limitations in sensitivity apply in the clinically relevant concentration range for congenital CMV disease (reported mean CMV DNA blood loads of 3.4[27], 4.0, and respectively 5.9

log_{10} copies/ ml in asymptomatic newborns with hearing loss at follow-up[28]). In this respect, it is important to note that defined clinically important CMV DNA loads, in the absence of an international CMV DNA quantification standard, are of use only in the laboratory setting where they were obtained. For a general application, standardization of CMV DNA values obtained by different PCR protocols and different quantification standards is essential.

Specificity of CMV DNA detection using DBS has been reported to range between 99.3% and 100%.[12,14,25] To our knowledge, transfer of CMV DNA from one DBS to another during storage has been reported once.[26] Transfer of CMV DNA during punching can be controlled for in the procedure (see below). However, both these potential contaminating events are not likely to be of practical significance given the above described limited analytical sensitivity.

Above mentioned advocated newborn screening for congenital CMV can only be achieved using automated, high-throughput DNA extraction methods. Currently, few methods appear suitable for 96-well format high-throughput testing.[15]

Here, we describe two methods for efficient extraction of DNA from DBS, used for CMV DNA detection.

Materials

DNA extraction from DBS using heat shock[12, 24]:

1. (Automated) paper puncher
2. Positive and negative control DBS (or blanc Guthrie card/ Whatman 903 filter paper) (*see Note 1*)
3. (Eppendorf) tubes or 96-well plate
4. Minimal Essential Medium (MEM, without additives)
5. Cooler or thermal cycler (4°C)
6. Heating block or thermal cycler at 55°C and subsequently 100°C
7. (Eppendorf table) centrifuge
8. Internal control to monitor for PCR inhibition (e.g. phocine herpes virus (PhHV) DNA)

Column-based DNA extraction from DBS:

1. (Automated) paper puncher
2. Positive and negative control DBS (or blanc Guthrie card/ Whatman 903 filter paper) (*see Note 1*)

3. Microcentrifuge tube
4. QIAamp DNA Mini Kit (containing columns, collection tubes, lysis buffer (ATL, AL), proteinase K, wash and elution buffer)
5. Heating block or water bath at 85°C, 56°C, and subsequently 70°C
6. Eppendorf table centrifuge
7. Internal control to monitor for PCR inhibition (e.g. phocine herpes virus (PhHV) DNA)

Methods

DNA extraction from DBS using heat shock.[12, 24]

1. For each test DBS (*see Notes 1 and 2*), punch one disk of 3 mm (1/8 inch) in diameter per tube or well, in triplicate. Punch one disk from a negative control DBS between each test DBS (*see Notes 1 and 3*).
2. Add 35 µLMEM, including internal control DNA (e.g. PhHV DNA, *see Note 4*) and spin the punches down (*see Note 5*).
3. Incubate at 4°C overnight (*see Note 6*).
4. Perform heat shock (e.g. in thermal cycler or heating block) according the following protocol (*see Note 7*):
   - -55°C at 60 min
   - -100°C at 7 min
   - -cool rapidly to 4°C
5. Centrifugate at 3,320 x g for 15 min, or at 8,960 x g for 1-3 min (*see Note 8*).
6. Transfer the supernatant (approximately 25 µL) to an empty tube or 96-well plate and freeze at -80°C for at least 1 h (*see Note 9*).
7. Thaw; the extract is ready to use for PCR[15].
8. Interpretation of PCR results of triplicates (*see Note 10*).

Column-based DNA extraction from DBS (*see Notes 11 and 12*)

1. For each test DBS (*see Notes 1 and 2*), punch one whole DBS (of approximately 1 cm in diameter, corresponding with approximately 50 µL dried blood) (*see Note 12*) in a microcentrifuge tube, in triplicates. Punch a negative control DBS between each sample (*see Note 1 and 3*).
2. Add 180 µL lysisbuffer (ATL), including internal control DNA (e.g. PhHV DNA, *see Note 4*) to each tube.
3. Incubate at 85°C for 10 min.

4. Add 20 µL proteinase K, vortex, and incubate at 56°C for 1h.
5. Add 200 µL lysisbuffer (AL), vortex, incubate at 70°C for 10 min.
6. Add 200 µL ethanol 96-100%, vortex.
7. Apply the mixture (approximately 600 µL) to column in a collection tube and centrifuge at 6,000 x g for 1 min, discard the filtrate.
8. Wash with 500 µL washbuffer (AW1) at 6,000 x g for 1 min, discard the filtrate.
9. Wash with 500 µL washbuffer (AW2) at 20,000 x g for 3 min, discard the filtrate.
10. Centrifuge once more at full speed for 1 min, discard the filtrate.
11. Elute the DNA with 150 µL elution buffer (AE) after incubation for 1 min and centrifugation at 6,000 x g for1 min.
12. The eluate is ready to use for PCR[15].
13. Interpretation of PCR results of triplicates (*see Note 10*).

Notes

1. Positive and negative control DBS can be produced by spotting CMV DNA positive and negative (EDTA) blood on Whatman 903 filter paper (approximately 50 µL per spot of 1 cm diameter) followed by air-drying. DBS can be stored at 4°C or at room temperature.
2. When dried on filter paper, blood spots are considered non-infectious material.
3. DNA contamination from sample to sample during punching is controlled for by testing a negative control DBS in between each test DBS.
4. PCR inhibition can be controlled for in a simultaneous reaction by adding a fixed amount of internal control (e.g. PhHV DNA) to each sample. Inhibition of internal control amplification is indicative of potential inhibition of amplification of target (CMV) DNA.

Notes specific for <u>heat shock</u> DNA extraction:

5. Punches must be spun down until the disks are below liquid surface level (15 min at 3,320 x g may be necessary when using a 96-well plate).
6. Incubation at 4°C overnight significantly enhances extraction efficiency.
7. During the heat shock, DNA will be extracted from the DBS.
8. Centrifugation yields sufficiently purified DNA.
9. Freezing the supernatant for at least 3 h enhances extraction efficiency (no maximum freezing time implicated).
10. Triplicate testing results in optimal sensitivity.[15] Interpretation of triplicate PCR results can be performed using the flow diagram as described by Barbi et al[13], in

which every positive result should be confirmed with at least one additional positive result, in the same run, or in case of a single positive test result (1 of the 3 replicates), by means of a confirmatory PCR procedure including DNA extraction (second run) (Figure).

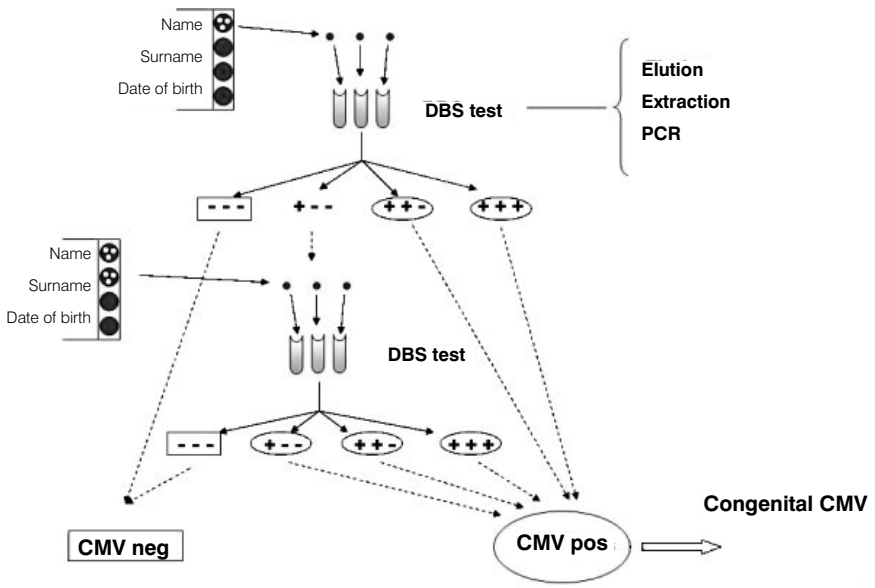

Figure Flow diagram for interpretation of triplicate testing results as proposed by Barbi et al[13].

Notes specific for column-based DNA extraction:

11. Detailed protocol is described in the manufacturers "QIAamp DNA Mini and Blood Mini Handbook", version April 2010.

12. Using one whole DBS (diameter of 1 cm, corresponding with approximately 50 µL blood) enhances sensitivity (DNA yield) significantly, when compared to three or six punches of 3 mm in diameter as proposed in the "QIAamp DNA Mini and Blood Mini Handbook" (version April 2010).

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Chapter 8

Rapid genotyping of cytomegalovirus in dried blood spots by multiplex real-time PCR assays targeting the envelope glycoprotein gB and gH genes

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Abstract

Genotyping of cytomegalovirus (CMV) is useful to examine potential differences in the pathogenicity of strains and to demonstrate coinfection with multiple strains involved in CMV disease in adults and congenitally infected newborns. Studies on genotyping of CMV in dried blood spots (DBS) are rare and have been hampered by the small amount of dried blood available. In this study, two multiplex real-time PCR assays for rapid gB and gH genotyping of CMV in DBS were developed. Validation of the assays with 39 CMV-positive plasma samples of transplant recipients and 21 urine specimens of congenitally infected newborns was successful in genotyping 100% of the samples, with gB1 and gB3 being the most prevalent genotypes. Multiple gB and gH genotypes were detected in 36% and 33% of the plasma samples, respectively. One urine sample from a newborn with symptomatic congenital CMV was positive for gB1 and gB2. DBS of congenitally infected newborns (n = 41) were tested using 9 µl of dried blood, and genotypes were detected in 81% (gB) and 73% (gH) of the samples, with gB3 being the most prevalent genotype. No clear association of specific genotypes with clinical outcome was observed. In conclusion, the CMV gB and gH PCR assays were found to be rapid, sensitive for detecting mixed infections, and suitable for direct usage on DBS. These assays are efficient tools for genotyping of CMV in DBS of congenitally infected newborns.

Cytomegalovirus (CMV) is the most common cause of congenital infection worldwide and an important viral pathogen affecting immunocompromised patients.[1,2] Both in congenitally infected newborns and in immunocompromised patients, genotyping of CMV has been used to study potential differences in pathogenicity of specific strains. However, few authors describe a correlation between specific CMV genotypes and severity of disease.[3-6] More important, genotyping of CMV has enabled the discrimination of reactivation of latent virus from reinfection with new CMV strains in transplant patients, allowing a better definition of donor-to-recipient transmission patterns.[7] Congenital CMV infections mainly result from recurrent infections among pregnant women[8], comprising both reactivation and reinfection. The discrimination of reactivation from reinfection may give insight into the mother-to-fetus transmission pattern and the possible associations with the outcome of congenital CMV infections. Genotyping of CMV has mainly focused on envelope glycoproteins gB (UL55) and gH (UL75), which play a role in virus entry and are major targets for neutralizing antibody response. The most frequently used methods for genotyping of CMV are nucleotide sequence analysis[9] and restriction fragment length polymorphism of PCR products.[10,11] Recently, real-time PCR-based assays have been used for rapid detection and quantification of CMV gB and gH genotypes.[7,12-14] However, they have mainly been applied to plasma or other high-volume samples. Also, deep-sequencing-based methods, sensitive in the detection of genotype mixtures with very low ratios, required a large input of CMV genomes.[15] Studies on genotyping of CMV in dried blood spots (DBS) are rare[16,17] and are hampered by the small amount of dried blood (50 µl per spot) available. In this study, two multiplex real-time PCR assays for rapid gB and gH genotyping of CMV were developed and applied to DBS obtained from congenitally infected neonates.

Materials and methods

Plasma samples of immunocompromised patients

A total of 39 CMV DNA-positive plasma samples (loads, ≥1,000 copies/ml) were randomly selected from the database of the Department of Medical Microbiology of the Leiden University Medical Center (time period, 2009 to 2011). The samples were from immunocompromised patients (median age, 50 years; range, 7 to 78 years): 26 stem cell transplant patients (of whom 22 were allogeneic), 11 kidney transplant patients, and 2 liver transplant patients (median CMV DNA load of 25,000 copies/ml; range, 1,000 to 25,000,000 copies/ml). The pretransplant donor/recipient (D/R)

CMV serostatus was distributed as follows: stem cell transplant patients, D^+/R^+ (n = 18), D^-/R^+ (n = 3), D^+/R^- (n = 1), and D^-/R^- (n = 1); kidney transplant patients, D^+/R^+ (n = 10), D^-/R^+ (n = 3), and D^+/R^- (n = 5); and liver transplant patient, D^-/R^+ (n = 1) (D/R serostatus was not available for 5 patients).

Urine samples from newborns with congenital CMV

Urine samples with control gB1 to gB4 strains (determined by means of restriction fragment length polymorphism[16]) were kindly provided by Maria Barbi, Department of Public Health-Microbiology-Virology, University of Milan, Milan, Italy. Furthermore, 21 CMV culture-positive urine samples from congenitally infected newborns (sampled within 3 weeks after birth) were derived from the database of the Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands (n = 19; time period, 2000 to 2011), and the Department of Medical Microbiology of the Leiden University Medical Center (n = 2; time period, 2009 to 2011), irrespective of clinical characteristics at birth (median CMV DNA load, 100,000 copies/ml; range, 4,000 to 20,000,000 copies/ml). No clinical data were available for the congenitally infected newborns tested and the CMV serostatus of the mother.

Dried blood spots (DBS) from newborns with congenital CMV

A total of 41 DBS from newborns with congenital CMV infection were obtained from earlier studies (median CMV DNA load of 5,000 copies/ml whole blood; range, <1,000 to 800,000 copies/ml). Nine of the 41 newborns participated in the previously described DECIBEL study, which included infants with permanent bilateral hearing impairment (≥40 dB in the better ear) at the age of 3 to 5 years (median CMV DNA load of 32,000 copies/ml whole blood).[18] Clinical data included symptoms at birth, developmental score, and severity of hearing loss. The remaining 32 CMV-positive DBS were derived from a prevalence study in which a random selection of DBS from the Netherlands (2007) was tested for CMV DNA (median load of 5,000 copies/ml whole blood).[19] Due to the anonymization of the samples, no clinical data were available from these 32 newborns.

DNA extraction from plasma and urine samples

Nucleic acids from plasma samples were extracted using the Cobas AmpliPrep total nucleic acid kit. Nucleic acids from urine samples were extracted on the MagNA Pure LC using the total nucleic acid isolation kit and high performance kit (both from Roche Diagnostics, Almere, the Netherlands). The input volumes were 350 µl plasma and 200 µl urine, and output volumes were 100 µl.

DNA extraction from DBS

DNA was extracted from DBS using the QIAamp DNA minikit according to the protocol for isolation of total DNA from FTA and Guthrie cards (Qiagen, Hilden, Germany). Sample input per well was 3 punches each measuring 3.2 mm in diameter, corresponding with approximately 9 µl dried blood per well. DBS were punched using an automated plate punch type 1296-071 (Perkin Elmer-Wallac, Zaventem, Belgium), with a negative-control punch between each sample. Output volume was 100 µl. DNA extraction was followed by CMV amplification in duplicate (DECIBEL DBS samples) or triplicate (DBS from prevalence study).

CMV gB-and gH-specific primers and probes

For the selection of primers and probes, an alignment of CMV gB and gH gene sequences available in GenBank was made using the AlignX program (Vector NTI Advance 11; Invitrogen). The accession numbers of gB and gH sequences that were used were as follows: CMV gB genotype 1, M60929, EF999921, GQ466044, GQ221974, AY446894, U66425, GQ121041, and FJ616285; gB genotype 2, GQ221975, X17403, FJ527563, BK000394, X04606, M60931, and M60932; gB genotype 3, M60934, M85228, and M60933; gB genotype 4, M60926 and M60924; CMV gH genotype 1, AB275152, AB275255, AJ239007, BK000394, EF999921, FJ527563, GQ396663, GQ466044, GU179290, and X17403; gH genotype 2, AB275156, AY446894, FJ616285, GQ121041, GQ221973, GQ396662, GU179291, and M94233. Subsequently, specific primers and probes were designed for efficient amplification of multiple genotypes in one reaction, supported by the software package Beacon Designer 7.91 (Premier Biosoft International, Palo Alto, CA). The sequences of primers and probes are summarized in Tables 1 (gB) and 2 (gH). The gB3-specific probe was published by Gorzer et al.[13]

Quantitative CMV real-time PCR

In the sensitivity analysis of the newly developed assays, our diagnostic real-time PCR was used to determine the CMV DNA load of the samples. Amplification of a 126-bp fragment from the CMV immediate-early antigen region was performed using an internally controlled quantitative real-time PCR as described previously.[20,21]
Quantification was performed using a dilution series of titrated CMV (strain AD169; Advanced Biotechnologies Inc., Columbia, MD) as an external standard.

Multiplex CMV gB1 to gB4 and gH1 and gH2 real-time PCR assays

CMV gB1 to gB4 and gH1 and gH2 specific DNA amplification was performed using two multiplex real-time PCR assays. Each multiplex assay contained 10 µl of DNA extract, 25 µl HotStar Master mix (Qiagen, Hilden, Germany), and final concentrations of 0.3 µM (all) specific forward and reverse primers (gB1 to gB4 or gH1 and gH2) (Tables 1 and 2), 0.2 µM (all) specific probes (gB1, gB2, gB3, gB4A, and gB4B or gH1 and gH2) (Tables 1 and 2), and 4.5 mM $MgCl_2$. Template denaturation and activation of HotStar *Taq* DNA polymerase for 15 min at 95°C were followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The PCR assays were carried out in a CFX96 real-time PCR detection system (Bio-Rad, Veenendaal, the Netherlands).

Table 1 Sequences of primers and probes for cytomegalovirus gB genotyping

Primer/probe name	Sequence (5'–3')	Product size (bp)
gB1 forward	TCA CCA TTC CTC TCR TAC GAC	93
gB1 reverse	CAC CAT GGC TGA CCG TTT GG	
gB1 TaqMan probe	FAM-TCT GCT GCT CAY TCT CGA TCC GGT TC–BHQ-1	
gB2 forward	CTT TAA GGT ACG GGT CTA CCA A	152
gB2 reverse	GAA CTG TAG CAT TGG GCA AAC T	
gB2 TaqMan probe	YAK-CTA CGC TTA CAT CYA CAC CAC TTA TCT GC–BHQ-1	
gB3 forward	CCG GTG TGA ACT CCA CGC G	73
gB3 reverse	GAT TCG CTT TCA RGY GAC AGG	
gB3 XS probe (15)[a]	TXR-TCG TAT TGC CCG TAC T–BHQ-2	
gB4 forward	TCG TGC AAC TTC TAC TCA TAA TG	85
gB4 reverse	CGT TAC GCG TTG AGA GGA GAT	
gB4 TaqMan probe A	Q705-AAA CCA TAC TTC TCA TAC GAC GTC TGC TC–BHQ-2	
gB4 TaqMan probe B	Q705-AAG CCA TAT TTC TCG TAC AAC GTC TGC TC–BHQ-2	

[a] XS probe, minor groove binding replacement probe.
Abbreviations: FAM, 6-carboxyfluorescein; BHQ, black hole quencher; YAK, Yakima Yellow; TXR, Texas Red.

Table 2 Sequences of primers and probes for cytomegalovirus gH genotyping

Primer/probe name	Sequence (5'-3')	Product size (bp)
gH1 forward	GAG ACT TAA CAC CTA CGC AT	181
gH1 reverse	CGA TCC CTT CCA GTC G	
gH1 TaqMan probe	FAM-GGG TCA GCA GCC CAC CAC C–BHQ-1	
gH2 forward	TGG ACA CGA TCT ACT ATT CA	134
gH2 reverse	TGT CGT CGT CTA TGG AC	
gH2 TaqMan probe	YAK-CAC CGT CAC ACC TTG TTT GCA CC–BHQ-1	

Abbreviations: FAM, 6-carboxyfluorescein; BHQ, black hole quencher; YAK, Yakima Yellow.

Results

Analytical sensitivity

The analytical sensitivities of the multiplex real-time CMV gB and gH PCR assays were determined using 10-fold dilution series of CMV-positive plasma samples with single genotypes gB1, gB2, gB3, and gB4 and gH1 and gH2, respectively. Comparison of the multiplex CMV gB and gH PCR assays with the diagnostic CMV PCR[21] resulted in equal detection limits of approximately 250 copies/ml.

Comparison of the results of the multiplex gB and gH PCR assays with the monoplex gB and gH PCR assays, testing the above-mentioned plasma samples, revealed comparable cycle threshold values (<1.5 cycle threshold difference) (data not shown).

Furthermore, plasma mixtures of CMV gB1-gB2, gB1-gB3, gB1-gB4, gB2-gB3, gB2-gB4, gB3-gB4, and gH1-gH2 were prepared, each combination in different ratios. The detection limit of the minor variant in these mixtures was approximately 250 CMV DNA copies/ml, which could be detected in mixtures with a proportion of the minor variant down to about 0.2% (data not shown).

The analytical sensitivity of the multiplex real-time CMV gB and gH PCR assays for DBS was determined using DBS with a broad range of CMV DNA loads (range, 50 to 20,000 copies/ml whole blood) (the Quality Control for Molecular Diagnostics (QCMD) CMV DBS 2011 panel and DBS samples prepared in-house with CMV-positive blood

from transplant recipients [20]). Comparison of the multiplex CMV gB and gH PCR assays with the diagnostic CMV PCR[21] using DBS resulted in equal detection limits of approximately 1,000 to 2,500 copies/ml (data not shown).

Good precision was observed in the multiplex real-time CMV gB and gH PCR assays. Replicates of DNA from each genotype (gB1 to gB4 and gH1 and gH2) run on different days resulted in a mean difference of cycle threshold values of 0.6 ± 0.4 (standard deviation [SD]) (range, 0 to 1.5) (data not shown).

Analytical specificity

The multiplex real-time CMV gB and gH PCR assays were negative for plasma and urine samples with noncorresponding gB1 to gB4 and gH1 and gH2 genotypes; no cross-reactions were observed. Furthermore, the assays tested negative for plasma samples with the genomes of Epstein-Barr virus, herpes simplex virus, and varicella-zoster virus.

Detection of CMV gB and gH genotypes in plasma samples of immunocompromised patients

A random selection of CMV DNA-positive plasma samples of 39 transplant patients was tested using the multiplex CMV gB and gH PCR assays (Fig. 1). All 39 samples could be assigned to gB and gH genotypes (median cycle threshold value, 32; range, 24 to 42). The most prevalent genotypes were gB1 (54%; 21/39) and gB3 (41%; 16/39).

Multiple CMV gB and gH genotypes were detected in 36% (14/39) and 33% (13/39), respectively, of the CMV-positive plasma samples (median CMV DNA load of 40,000 copies/ml versus 25,000 copies/ml in single infections). Of these mixed gB infections, 28% (11/39) were double and 8% (3/39) were triple infections. Double gB genotype infections included gB1-gB2 (n = 4), gB1-gB3 (n = 4), gB2-gB3 (n = 2), and gB1-gB4 (n = 1). Triple gB/gH genotype infections included gB1-gB3-gB4/gH1-gH2 (n = 1 stem cell transplant, D^+/R^+), gB2-gB3-gB4/gH1-gH2 (n = 1 kidney transplant, D^-/R^+), and gB1-gB2-gB3/gH1-gH2 (n = 1 kidney transplant, D^-/R^+). The pretransplant CMV serostatuses of single compared to mixed gB infections were not significantly different (single gB: D^+/R^+, n = 12; D^-/R^+, n = 5; D^+/R^-, n = 5; mixed gB: D^+/R^+, n = 8; D^-/R^+, n = 2; D^+/R^-, n = 1; D^-/R^-, n = 1).

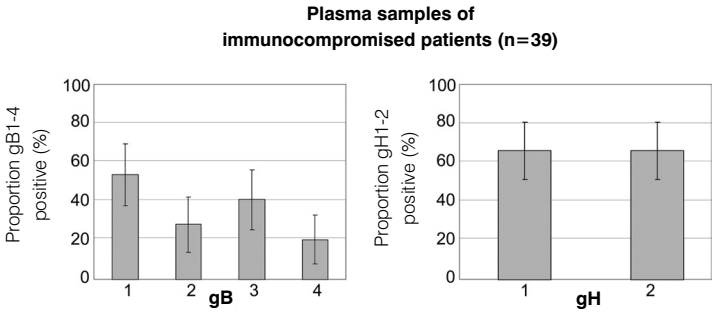

Figure 1 Distribution of CMV gB and gH genotypes (%) detected in CMV DNA-positive plasma samples of immunocompromised patients (n = 39). Note that the percentages add up to more than 100% due to the detection of multiple gB and gH genotypes in 36% (14/39) and 33% (13/39), respectively, of the CMV-positive plasma samples, with double and triple gB types in 28% (11/39) and 8%, respectively, of the samples. Error bars represent the 95% confidence interval. CMV, cytomegalovirus.

Detection of CMV gB and gH genotypes in urine samples from newborns with congenital CMV

Urine samples obtained from 21 newborns with congenital CMV infection were tested using the multiplex real-time gB and gH assays (Fig. 2). A genotype could be assigned to all urine samples, with CMV gB1 (48%, 10/21), gB3 (29%, 6/21), and gH2 (62% 13/21) being the most prevalent genotypes (median cycle threshold value, 27; range, 21 to 36). One urine sample of a newborn with symptomatic congenital CMV was positive for both gB1 and gB2 (and gH2), indicating a mixed congenital infection. Clinical data revealed that this newborn was severely symptomatic at birth, with microcephaly, hyperbilirubinemia, thrombocytopenia, petechiae, and hepatosplenomegaly, and at a later age was diagnosed with mental retardation and hearing impairment. No clinical data were available from the other congenitally infected newborns tested.

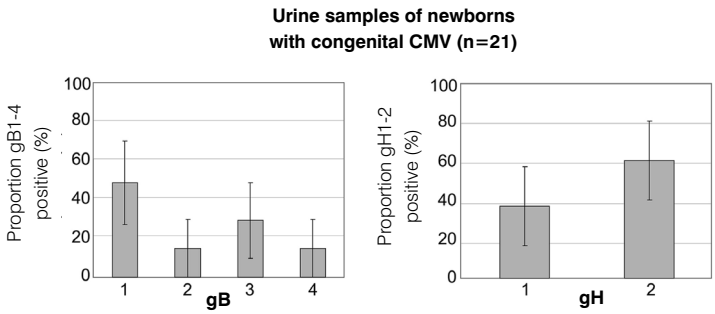

Figure 2 Distribution of CMV gB and gH genotypes (%) detected in CMV positive urine samples (<3 weeks of age) of congenitally infected newborns (n = 21). Error bars represent the 95% confidence interval.

Detection of CMV gB and gH genotypes in dried blood spots

Dried blood spots (DBS) from 41 newborns with congenital CMV infection were tested in the multiplex real-time CMV gB and gH assays (Fig. 3). In total, 33 (81%) and 30 (73%) of the 41 DBS could be assigned a gB and gH genotype, respectively (median cycle threshold value, 36; range, 29 to 39). The most prevalent genotype was gB3 (32%, 13/41). The gH genotypes were distributed evenly.

Clinical data were known for 9 of the 41 newborns and are shown in Table 3. These children had permanent bilateral hearing impairment at the age of 3 to 5 years, since that was an inclusion criterion for participation in the DECIBEL study from which they were recruited.[18] A genotype could be assigned to 7 of the 9 (78%) DBS from children with hearing impairment. Genotype gB1 was not detected in DBS of these infants with hearing impairment. All 3 newborns with symptoms at birth had CMV loads of 200,000 copies/ml or higher and were genotyped as gB3/gH2, gB2/ gH1, and gB4/gH1, respectively. No clear differences were seen in the gH and gB genotype distributions between the CMV-positive DBS from the children with hearing impairment and the DBS from the prevalence study.

Table 3 Detection of CMV gB and gH genotypes in DBS from children with hearing impairment at the age of 3 to 5 years[18]

DBS from newborn	gB genotype	gH genotype	CMV IE load in DBS (log10 copies/ml whole blood)	Symptom(s) at birth	Developmental score[a]	Severity of hearing loss
1	3	2	4.1	None	59	60–90 dB
2	ND	ND	3.3	None	84	40–60 dB
3	4	1	4.9	None	72	>90 dB (CI)
4	2	2	3.6	None	68	60–90 dB
5	3	2	5.9	IUGR, microcephaly, seizures	30	>90 dB (CI)
6	2	1	5.3	IUGR, petechiae, hepatosplenomegaly, jaundice, thrombocytopenia	50	>90 dB (CI)
7	4	1	5.3	IUGR, jaundice, microcephaly	Unknown	>90 dB
8	2	1	<3.0	None	82	>90 dB (CI)
9	ND	ND	3.6	Cataract	56	>90 dB (CI)

[a] The general development score is a summary score that provides an overall index of development by including 10 of the most age-discriminating items from each scale of the Child Development Inventory. The Child Development Inventory is a standardized instrument (parent questionnaire) designed to assess the social development, language development, and motor development of young children. Higher scores indicate better development.

Abbreviations: IE, immediate-early antigen; DBS, dried blood spots; gB, glycoprotein B; gH, glycoprotein H; CI, cochlear implant; IUGR, intrauterine growth retardation; ND, not detected.

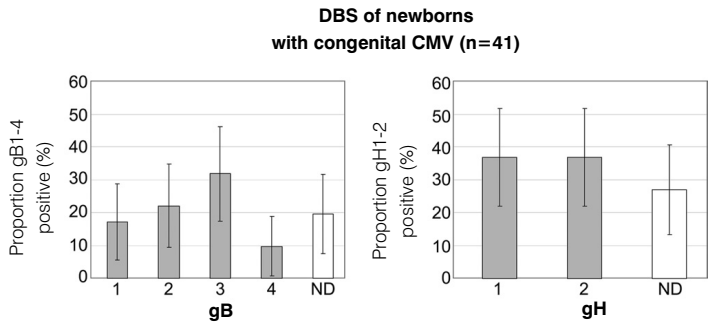

Figure 3 Distribution of CMV gB and gH genotypes (%) detected in CMV positive dried blood spots (DBS) of newborns with congenital CMV (n = 41). Error bars represent the 95% confidence interval. ND, not detected.

Discussion

In this study, two multiplex real-time PCR assays were used for rapid CMV gB and gH genotyping on DBS. Validation of these gB1 to gB4 and gH1 and gH2 specific PCR assays showed excellent sensitivity for genotyping plasma samples of immunocompromised patients and urine samples of congenitally infected newborns. Furthermore, the assays were able to detect a high number of mixed infections (>30%) in CMV-positive plasma samples and in one urine sample of a severely symptomatic newborn. In DBS of congenitally infected newborns, using only 9 µl of dried blood, a CMV genotype could be determined in 81% (gB) and 73% (gH), respectively, of the cases.

Our finding that genotypes gB1 and gB3 were the most prevalent genotypes in immunocompromised patients and congenitally infected infants is in agreement with previous studies assessing the genotype distribution of CMV.[13,14,16,22-27] Potential significant variances in genotype distribution found in different studies are potentially based on geographical distribution, the population of patients tested, and/or CMV tissue tropism. In our study, no significant differences were found between the genotype distributions as detected in the urine samples, which were taken from mainly hospitalized newborns, and the DBS, which were from a different group of newborns, including a selection of children with hearing loss at the age of 3 to 5 years. It must be noted that the number of congenitally infected newborns tested in our study is small,

and therefore, we cannot exclude differences in genotype distribution. Potential differences might be based on the population of newborns tested (symptomatology/hearing impairment), and also a slight difference in sensitivity between the gB1 and gB3 assays cannot be excluded (due to the lack of genotype-specific standards).

We could detect mixed-genotype infections in >30% of plasma samples from immunocompromised patients. It must be mentioned that potential mixed infections with viral loads below the detection limit could be missed and, therefore, the actual proportion of mixed infections might even be higher. The high proportion of mixed infections detected in our study is comparable to or exceeds the proportion found in previous studies, with a mixed genotype detected in 15 to 21% of the (solid organ) transplant recipients.[7,12-14,28] This would suggest that our assays were at least as sensitive. The risk of competitive amplification of multiple genotypes by generic primers has been reduced by using genotype-specific primers and probes. This method was found to be more sensitive for detecting mixed infections (data not shown). Furthermore, the high proportion of D^+/R^+ and stem cell transplant recipients in our study might also contribute to the high proportion of mixed infections detected, since the highest genotype diversity has been found in these populations.[15] It has been demonstrated that the CMV load after transplantation reflects the sum of relative levels of individual genotypes in time.[13] Mixed CMV genotypes could be detected significantly more often in patients with higher CMV loads than in patients with lower loads[14], though the interpretation might be biased by underdetection of mixed infections with low viral loads. In this way, an association of mixed infections (and corresponding higher CMV loads) with clinical outcome has been demonstrated in transplant patients. [22,29,30]

The occurrence of mixed congenital CMV infections in live newborns has rarely been described before. Though coinfection with multiple gB genotypes has been reported in two postpartum mothers of congenitally infected infants[26], one report casually noted the detection of mixed gB types in urine specimens from two congenitally infected newborns[31], and another report suggested the presence of multiple US28 and UL144 genotypes in 8 of 10 autopsy tissues from fatal cases of congenital CMV infection.[32] The association of mixed infections with severe disease found in immunocompromised patients, combined with the single report on frequent mixed congenital CMV infections in fetal deaths, may lead to the speculation that congenital CMV infections by multiple strains (correlated with higher viral loads) could be associated with severe symptomatology and possibly fetal death. Interestingly, very recent genome-wide next-generation sequencing of CMV present in urine of three

congenitally infected newborns revealed mixed gB and gN genotypes and offered strong evidence that CMV exists as a complex mixture of genome variants, with intrahost variability (0.2%) comparable to that of many RNA viruses.[33]

In agreement with earlier studies that attempted genotyping CMV on DBS, a genotype could not be assigned to all strains from positive DBS.[16,17] Detection of CMV DNA in DBS has been shown to be a challenge [20,34-40] due to the small amount of dried blood (50 µl per spot) available. Optimizing the DNA extraction step from the DBS has been shown to result in significantly increased sensitivity of CMV DNA detection in DBS. The DNA extraction method used in this study has been optimized previously, and despite a limited input of only 9 µl dried blood per well, a genotype could be determined in approximately 75 to 80% of the DBS samples. Recently developed deep-sequencing methods[15] have been reported to be highly sensitive because of their ability to detect genotype mixtures in low ratios, but they require a large input of approximately 280 µl of whole blood (30 times more than that used in our assay) containing 50,000 to 500,000 CMV DNA copies/ml plasma.[15] For comparison, the median CMV DNA whole-blood load in congenitally CMV-infected newborns (symptomatic and asymptomatic newborns) has been reported to be 2,300 copies/ml blood.[41]

The association of specific CMV genotypes with congenital CMV disease has previously been addressed with controversial results and is limited to the association of genotype gN4 with long-term sequelae[3] and genotype gB3 being found more often among congenitally CMV-infected than in postnatally infected children.[6] In our study, no clear association between specific CMV gB and gH genotypes and severity of disease was observed, though the sample numbers were low.

Genotyping of CMV has been shown to enable the discrimination of reactivation of latent virus from reinfection with new CMV strains in transplant patients and has enabled the assessment of donor-to-recipient transmission patterns.[7,28] Data from Manuel et al. suggest that, in seropositive transplant recipients, approximately half of the infecting CMV strains originate from the organ donor and the other half are reactivated endogenous strains.[7] Though CMV is more frequently transmitted to the fetus in preconceptionally seronegative women, recent calculations have demonstrated that the majority of congenitally CMVinfected children in the United States are born from seroimmune women.[8] This major role of recurrent maternal infections emphasizes the convenience of a sensitive and rapid CMV genotyping assay, suitable for usage on DBS, in order to compare potentially mixed genotypes present in maternal blood with CMV strains in the newborn. The rapid and sensitive genotyping tool described in this study may support a better definition of mother-

to-fetus transmission patterns and may lead to enhanced insight into transmission risk and outcome of congenital CMV infections. The implications of this increased insight into transmission risks for preventive and therapeutic strategies, including CMV vaccine research, may be significant.

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Chapter 9

Real-time PCR versus viral culture on urine as a gold standard in the diagnosis of congenital cytomegalovirus infection

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Abstract

Background

Cytomegalovirus (CMV) infection is the most common cause of congenital infection. Whereas CMV PCR has replaced viral culture and antigen detection in immunocompromised patients because of higher sensitivity, viral culture of neonatal urine is still referred to as the gold standard in the diagnosis of congenital CMV infection.

Objective

To compare real-time CMV PCR with shell vial culture on urine in the diagnosis of congenital CMV, in a multicenter design.

Study design

A series of neonatal urines (n = 340), received for congenital CMV diagnostics and routinely assessed with shell vial CMV culture, was retrospectively tested by real-time CMV PCR.

Results

The proportion of newborns found to be congenitally infected by real-time CMV PCR was 8.2% (28/340, 95%CI 5.6–11.8%), and 7.4% (25/340, 95%CI 4.9–10.8%) by rapid culture. When considering rapid culture as reference, real-time PCR was highly sensitive (100%), whereas sensitivity of rapid culture was 89.3% when considering real-time PCR as reference.

Conclusions

Our results, supported by analytical and clinical data on CMV DNA detection in neonatal urine, suggest enhanced sensitivity of recent PCR techniques when compared to viral culture. There is considerable rationale to favor real-time CMV PCR as a gold standard in the diagnosis of congenital CMV infection. A large-scale study combining both laboratory and clinical data is required to determine the exact time frame for sampling of neonatal urine when using real-time PCR.

Background

Cytomegalovirus (CMV) infection is the most common cause of congenital infection and a leading cause of non-genetic sensorineural hearing loss.[1–3] For decades, the gold standard in the diagnosis of congenital CMV infection has been viral culture of urine, sampled within the first 2 or 3 weeks of life.[4–6] After this period, CMV present in urine may be due to postnatally acquired infection. Meanwhile, PCR assays have been optimized by improved extraction and amplification techniques (e.g. real-time detection and internal controls for PCR inhibition), resulting in highly sensitive and specific assays. CMV DNA detection has become a routine diagnostic tool at many centers thanks to its rapid, reproducible, automated and quantitative nature.[7,8] Experiments with dilution series have shown that the analytical sensitivity of CMV PCR on urine is approximately 100 times higher than both traditional tube and shell vial culture.[9] In immunocompromised patients, CMV PCR has replaced CMV blood culture and pp65 antigen detection because of the higher sensitivity.[10–12] Furthermore, the clinical sensitivity of CMV PCR on urine of kidney and liver transplant patients is higher than viral culture,[13–15] with CMV DNA loads in urine being predictive of CMV disease.[11,16,17]Strikingly, in recent guidelines and reviews on congenital CMV, viral culture of neonatal urine remains referred to as the gold standard for confirmatory diagnosis, while CMV PCR is mentioned as plausible alternative more frequently.[18–20]

Objective

The aim of this study was to compare real-time CMV PCR with shell vial culture on urine in the diagnosis of congenital CMV, in a multicenter design.

Study design

Urine samples

A series of neonatal urines, sampled within the first 3 weeks after birth, received for congenital CMV diagnostics and routinely assessed with shell vial CMV culture,[21] was retrospectively tested by real-time CMV PCR.[22–24] All CMV culture positive samples ($n = 25$) and a large random selection of CMV culture negative urine samples ($n = 315$) dating from 2001 to 2011, were included in the analysis, irrespective of clinical characteristics of the newborns. All diagnostic urine samples were stored at -80 °C in the Dutch participating laboratories until tested by real-time PCR at

that specific site (Leiden University Medical Center (LUMC, n = 61 urines), Erasmus Medical Center Rotterdam (Erasmus MC, n = 199 urines), and Academic Medical Center Amsterdam (AMC, n = 80 urines)). Because of ethical reasons, retrospective testing was performed anonymously.

Viral culture and real-time PCR

Shell vial culture and CMV DNA extraction followed by amplification using seal herpesvirus (PhHV-1) as internal PCR control were performed as described previously.[21–24] In short, extraction was performed on the MagnaPure LC station using the Total Nucleic Acid Isolation Kit – High Performance Kit (both Roche Diagnostics, Almere, The Netherlands) (all sites), and the PCR was carried out using a CFX96 TM real-time PCR detection system (BioRad, Veenendaal, The Netherlands) (LUMC)/a LightCycler480 PCR system (Roche Diagnostics, Almere, The Netherlands) (Erasmus MC, AMC). Amplified was a 126-bp fragment of the CMV immediate early antigen region (LUMC, AMC)/a 133-bp fragment of the CMV DNA polymerase gene (Erasmus MC).

Statistical analysis

Sensitivity and specificity were calculated with both rapid CMV culture and real-time CMV PCR as reference. Kappa was calculated to assess test agreement.

Results

In total 340 urine samples of newborns ≤3 weeks of age were included in the comparison and were retrospectively tested with CMV real-time PCR (Table 1). The proportion of newborns found to be congenitally infected by rapid culture was 7.4% (25/340, 95%CI 4.9–10.8%), and 8.2% (28/340, 95%CI 5.6–11.8%) by real-time CMV PCR. All culture positive samples were detected by CMV PCR. In contrast, three urine samples were detected by real-time PCR that were negative in rapid CMV culture. When considering rapid culture as reference, real-time PCR was highly sensitive (100%) and specific (99.1%). Sensitivity of rapid culture was 89.3% when considering real-time PCR as reference. The CMV DNA load of the three samples with discrepant results (median 64,000 copies/ml, range 24,000–210,000 copies/ml, Table 2) was lower than the load of the 25 culture-positive samples (median 260,000 copies/ml, range 4400–95,000,000 copies/ml). These three urines were sampled at day 10, 17 and 17 of age, respectively, whereas the median time of sampling of the 25 culture-positive samples was 3 days (range 0–11 days). Additional testing of the discrepant

samples by repeated extraction and amplification of a different target (gB) gene (at a different participating center) yielded confirmatory positive PCR results.

Table 1 Comparison of internally controlled real-time PCR with shell vial culture of urine samples from newborns (n = 340) in the diagnosis of congenital CMV infection.

	Real-time CMV PCR		
	Positive	Negative	Total
Rapid CMV culture			
Positive	25	0	25 (7.4%)
Negative	3	312	315
Total	28 (8.2%)	312	340
Reference: real-time PCR			
Sensitivity culture (95% CI)	89.3% (70.6–97.2%)		
Specificity culture (95% CI)	100% (98.5–100%)		
Reference: rapid CMV culture			
Sensitivity PCR (95% CI)	100% (83.4–100%)		
Specificity PCR (95% CI)	99.1% (97.0–99.8%)		
Kappa (95% CI)	0.94 (0.87–1)		

Table 2 CMV DNA load and time of sampling of the urines with discrepant test results.

Rapid CMV culture	Real-time CMV PCR (copies CMV DNA/ml)	Time of urine sampling (days after birth)
Negative	Positive (24,000)	17
Negative	Positive (64,000)	17
Negative	Positive (210,000)	10

Discussion

In our multicenter comparison, CMV was more frequently detected in urine samples of newborns by real-time PCR than by rapid culture, which is still referred to as the reference method for diagnosing congenital CMV infection. These discrepant test results theoretically can be attributed to either false negative viral culture results, or false positive real-time PCR results. False negative viral culture results have been described both in experimental setting[9] and in clinical setting, testing urine samples of (immunocompromised) patients.[13–15,24] Loss of viable CMV particles implicated in false negative culture results may be caused by transport at room temperature[25] and antiviral therapy. In our analysis, two of the three discrepant samples had transport

times of >1 day. Concerning potential false positive PCR results, the use of real-time PCR procedures (which are less prone to contamination than nested procedures), the use of negative controls and confirmatory testing of the discrepant samples, render false positive CMV DNA detection in our study highly unlikely. Hence, false negative results in the viral culture assays constitute the most likely explanation for the discrepant test results in our study.

Previous studies comparing viral culture with CMV PCR on urine as initial or screening assay in the diagnosis of congenital CMV were reviewed, and sensitivity and specificity was calculated with both rapid CMV culture and real-time CMV PCR as reference (Table 3). Assuming PCR as reference, sensitivity of viral culture ranged from 61.5% to 100%. Negative CMV PCR results of culture positive urine samples were only described in earlier studies in which per report several gel-based PCR assays were compared while optimizing sensitivity.[26,27] Internal controls for potential PCR inhibiting components present in urine[28,29] were lacking in these studies.

Two of the three samples with discrepant test results in our study were taken 17 days after birth, and we cannot exclude that these were derived from postnatally acquired CMV infections. Postnatal CMV infection commonly occurs, because of frequent acquisition of CMV in the birth canal or from breast milk.[6] The restriction of samples taken within the first 3 weeks of life is considered safe to demonstrate congenitally acquired CMV infection.[5,6] However, literature contains mixed references to this time frame,[4,5,18,20] which is based on viral culture techniques and might not necessarily be identical for more sensitive diagnostic methods. Previous data have shown that CMV replicates with a doubling time of approximately one day.[30] Assuming that the analytical sensitivity of CMV PCR on urine is 100 times more sensitive than viral culture,[9] PCR could theoretically detect CMV 6.6 days (100 $\log_2$) earlier than viral culture, hence on day 14 postpartum compared to day 21 when using viral culture. Unfortunately, partially because of ethical reasons, neither clinical data nor other materials (saliva, (dried) blood) could be retrieved from these three newborns to discriminate congenital and postnatal infection. Data from studies combining clinical data with real-time CMV PCR results should address the distinction between congenitally and postnatally acquired CMV infection.

In conclusion, our results are supported by with analytical and clinical data on CMV DNA detection in neonatal urine and suggest enhanced sensitivity of recent PCR techniques. There is considerable rationale to favor real-time CMV PCR as a gold standard in the diagnosis of congenital CMV infection. A large-scale study combining laboratory and clinical data is required to determine the exact time frame for sampling of neonatal urine when using real-time PCR.

Table 3 Reports with data on both viral culture and CMV PCR on urine as initial or screening assay for diagnosing congenital CMV. Sensitivity and specificity of both viral CMV culture and CMV PCR were (re-)calculated with as reference method viral culture and CMV PCR, respectively. IC; inhibition control, cCMV; congenital CMV.

Reference [publication year]	Newborns included (age at sampling)	PCR method	Culture method[a]	Proportion CMV positive[b]	Reference: CMV PCR (%)		Reference: viral culture (%)	
					Sensitivity culture	Specificity culture	Sensitivity PCR	Specificity PCR
Present study	Suspected cCMV (<3 weeks)	Real-time, IC	Shell vial	8.2% (28/340)	89.3	100	100	99.1
31 [2011]	Suspected cCMV (<3 weeks[c])	Real-time,[d] IC	Shell vial	3.8% (10/260)	100	100	100	100
32 [2010]	Screened newborns (<2 weeks)	Real-time, IC	Shell vial	3.3% (3/91)	66.7	100	100	98.9
33 [2005]	Suspected cCMV (<3 weeks[c])	Nested,[d] IC, gel-based	Shell vial	12.6% (32/254)	100	100	100	100
34 [2001]	Screened newborns (<3 weeks)	Multiplex, gel-based	Tube	2.1% (7/332)	100	100	100	100
35 [2000]	Suspected cCMV (<2 weeks)	Real-time	Shell vial	75.0% (9/12)	77.8	100	100	60
27 [1996]	Screened newborns (<3 days)	Gel-based[e]	Tube	1.8% (18/1000)	100	99.9	94.4	100
26 [1994]	Suspected cCMV (majority), postnatal, immunocompromised	Gel-based[e]	Shell vial	36.9% (52/141)	61.5	97.8	94.1	81.3
36 [1988]	Screened newborns	Dot blot-based[e]	Tube	62.0% (44/71)	100	100	100	100

[a] Shell vial culture included staining with CMV (early) antigen-specific MAbs.
[b] Based on the number of PCR positive samples, with the exception of [27].
[c] 241 out of 254 newborns were sampled <3 weeks of age.
[d] Pool testing.
[e] Multiple PCR assays per study; data from the optimized PCR with improved sensitivity were used in the calculations.

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Chapter 10

Summarizing discussion

Summarizing discussion

This thesis addresses several aspects of congenital cytomegalovirus (CMV) infection in general and more specifically in the Netherlands, in order to determine the necessity and feasibility of newborn screening for congenital CMV. The major topics studied were

I. the **disease burden** of congenital CMV infection in the Netherlands,

II. postnatal **screening tools** for congenital CMV, and

III. pros and cons of **newborn screening** for congenital CMV.

In this chapter, the implications of our main findings are discussed, overall conclusions are formulated and recommendations for future studies are made.

PART I DISEASE BURDEN OF CONGENITAL CMV INFECTION

The birth prevalence of congenital CMV in the Netherlands was 0.54% (95%CI 0.36–0.72%) (*Chapter 2*)

IMPLICATIONS OF THIS FINDING

In a cross-sectional study, a large sample of dried blood spots (DBS) from infants born in the Netherlands was retrospectively tested for CMV DNA. The birth prevalence of congenital CMV was estimated at 0.54% (95%CI 0.36–0.72%) (*Chapter 2*). This finding, combined with the total number of newborns in the Netherlands (182,765 newborns/ year, 2007)[1], implicated that annually about 1000 children are born with congenital CMV infection in the Netherlands. This annual number of congenitally infected newborns is higher than some other well-known congenital conditions (Figure 1), including Down syndrome and spina bifida, for which prenatal screening is standard care.[2] Moreover, congenital CMV is at least 10 times more frequent than congenital hypothyroidism, and 100 times more frequent than homocystinuria, both disorders for which postnatal screening is standard care nowadays.[3] Based on the current knowledge on the natural history of congenital CMV infection[4], about 125 of these 1000 congenitally infected cases are expected to be symptomatic at birth. Approximately 5 congenitally infected newborns are expected to die each year in the Netherlands because of severe CMV inclusion disease. About 18% (1 out of 5) of the newborns with congenital CMV will develop neurological sequelae.[4] This implies that annually about 180 of these 1000 infected children born in the Netherlands will

eventually suffer from CMV-related sequelae, of whom 87%[4] (157) were asymptomatic at birth. The most frequently encountered sequela related to congenital CMV infection is hearing loss, followed by mental retardation, developmental delay, visual impairment, seizures, and paresis/paralysis. These conditions are known to have profound and life-long impact on the affected children and their families.

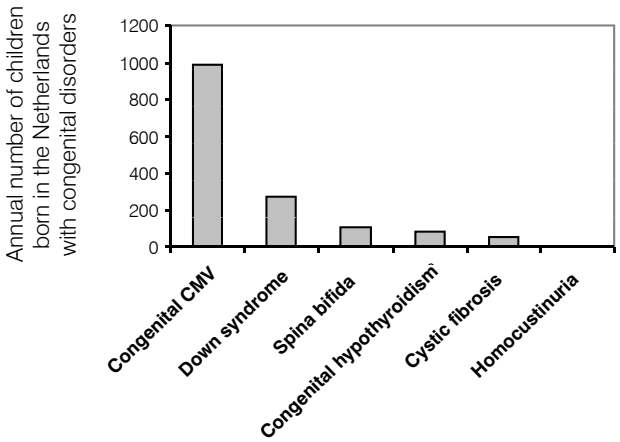

Figure 1 Annual number of newborns with congenital CMV in the Netherlands compared with several other congenital conditions (data from 2007).[2]

Recommendations for future studies

Our calculations of the number of infants with CMV-related sequelae and symptoms at birth were based on previous data on the natural history of congenital CMV infection in the United States.[4] The frequency and severity of clinical symptoms at birth and the long-term sequelae are known to differ among primary and non-primary maternal infections.[5] The proportion of primary and non-primary infections is associated with the seroprevalence in the underlying population (*Chapter 4*), and therefore varies among different countries. Thus, it would be interesting to study the prevalence of CMV-related symptoms and sequelae in the Netherlands, in a prospective study design. Follow-up of neurologic sequelae would be desirable for many years after birth because of the frequent late-onset and progressive nature of the hearing loss associated with congenital CMV.[6,7] Since developmental disorders (e.g. IQ<70) and visual impairment are the second and third most frequently encountered sequelae of congenital CMV infection[5,8], it would be interesting to address the prevalence of

congenital CMV-related mental retardation and more subtle mental, developmental and visual impairment in the Netherlands.
It has been suggested that congenital CMV infection is associated with disorders belonging to the autism spectrum. However, evidence is limited to case reports and a small series of children diagnosed with both autism and congenital CMV.[9] A large study would be necessary to rule out or confirm this speculative association of congenital CMV with autism. One of the major challenges of a retrospective analysis would be the age of diagnosis of autism, in combination with the limited time-frame to retrospectively diagnose congenital CMV using DBS (the storage duration of DBS in the Netherlands is 5 years).

About 1 out of 5 deaf children in the Netherlands was congenitally infected with CMV

2 of the 8 (25%) congenitally infected children with hearing loss at later age had passed the newborn hearing screening (*Chapter 3*)

Implications of these findings

Analyzing a cohort of children in the Netherlands with bilateral hearing loss at a later age (3-5 years), we found that the hearing loss was associated with congenital CMV infection in 1 in 5 deaf children (*Chapter 3*). This would render CMV the leading cause of non-genetic congenital hearing loss. Importantly, 2 of the 8 (25%) infants with both congenital CMV and hearing loss had passed the newborn hearing screening test, probably because of delayed-onset or progressive hearing loss. One should be aware that, in the absence of universal screening for congenital CMV infection, up to half [6] of the children with congenital CMV associated hearing loss at later ages may be missed by newborn hearing screening. Consequently, the Joint Committee on Infant Hearing recommended additional hearing evaluations in children with congenital CMV.[10]

Furthermore, we found that children with both hearing loss and congenital CMV had a greater delay in language comprehension than uninfected infants with comparable degrees of hearing loss. This implies that the delay in language comprehension in the infected infants was the result of a factor additional to the hearing loss, possibly cerebral damage resulting from congenital CMV infection.

Recommendations for future studies

The delayed onset and progressive nature of the hearing loss associated with congenital CMV is remarkable and the pathological mechanisms involved are largely unknown. CMV DNA has been detected in inner ear fluids (perilymph) of congenitally infected children up the age of 7 years, undergoing cochlear implant surgery.[11,12,13,14] The presence of CMV genome in the cochlea up to several years after birth supports the hypothesis of ongoing replication of CMV in the inner ear.[14] Moreover, this would be in line with data on long-term viral shedding in other body fluids of children with congenital CMV infection. The median duration of shedding of CMV in urine has been found to be approximately 4 years in both symptomatic and asymptomatic children.[15] CMV detection in the inner ear is limited to few reports describing a small number of congenitally infected patients. It would be interesting to further unravel the pathological mechanism of hearing loss associated with congenital CMV infection by analyzing the inner ear fluid of a large number of children undergoing cochlear implant surgery. Such a study would also provide insight in the proportion of congenital CMV infections among children with cochlear implants in the Netherlands, and would enable more detailed estimates of the disease burden and costs involved.

Subpopulations in the Netherlands with more young children, and with more non-western immigrants, had a higher risk of congenital CMV infection (*Chapter 2*)

Implications of this finding

Our region based case-control analysis showed that congenital CMV infection was most frequent in subpopulations with a high proportion of young children (a 6 times higher risk), and non-Western immigrants (a 3 times higher risk) (*Chapter 2*). The proportions of young children and immigrants in a population can be seen as demographic markers for environmental factors and behaviors that facilitate CMV transmission. Young children shed CMV in their body fluids, and a CMV shedding child

is a known risk factor for maternal CMV infection.[16] Similarly, CMV seroprevalence is reported to be higher among immigrant mothers than among native Dutch mothers[17], implicating a more frequent exposure to CMV. Factors involved in increased exposure and potentially related to cultural differences include large household size, crowding, certain child care practices, and possibly sexual practices.[8] Assessing subpopulations and indicating (behavioral) risk factors for congenital CMV infection in the Netherlands will provide insight in the transmission of CMV and potential preventive measures. While a vaccine is currently unavailable, prevention of congenital CMV infection is limited to hygiene practices.

Recommendations for future studies

In our study, risk factors were analyzed at a regional level. It would be interesting to assess risk factors for congenital CMV in the Netherlands at the individual level, in a prospective or retrospective design. Identification of risk factors is vital for proposing preventive measures. While there is evidence that hygiene counseling results in a reduced rate of CMV seroconversion among pregnant women[18,19,20], further studies are required to determine whether these measures reduce the rate of congenital CMV infection and disease.

Non-primary maternal CMV infections were estimated to account for the majority of congenital CMV infections (*Chapter 4*)

Implications of this finding

Applying the population-based prediction model we developed, we found that, for populations with CMV seroprevalence of 30% to 95%, non-primary maternal CMV infections accounted for the majority of congenital CMV infections (*Chapter 4*). The proportion of newborns with congenital CMV attributable to non-primary maternal infections was up to 96% in populations with seroprevalence of 95% (95%CI 88-99%). Additionally, the proportion of newborns with sequelae attributable to non-primary infections increased with CMV seroprevalence, and was up to 89% (95%CI 26-97%). These findings stressed the impact of non-primary infections on the disease burden of congenital CMV.

Combining this prediction model (*Chapter 4*) with our findings on the birth prevalence of congenital CMV in the Netherlands (*Chapter 2*), additional estimates could be made on the proportion and number of congenitally infected children born

from seropositive mothers in the Netherlands (Figure 2). Data on maternal CMV seroprevalence in the Netherlands (50%[17,21]) were combined with the annual number of newborns with congenital CMV and CMV-related sequelae in the Netherlands (987 and 177, respectively, *Chapter 2*). This resulted in an estimate of 681 congenitally infected children born from seropositive mothers in the Netherlands annually, of whom about 76 children eventually will be affected by sequelae. The birth prevalence of congenital CMV in the Netherlands as predicted by our model (based on 50% CMV seroprevalence) corresponded with the birth prevalence as detected in our cross-sectional study (0.51% and 0.54%, respectively).

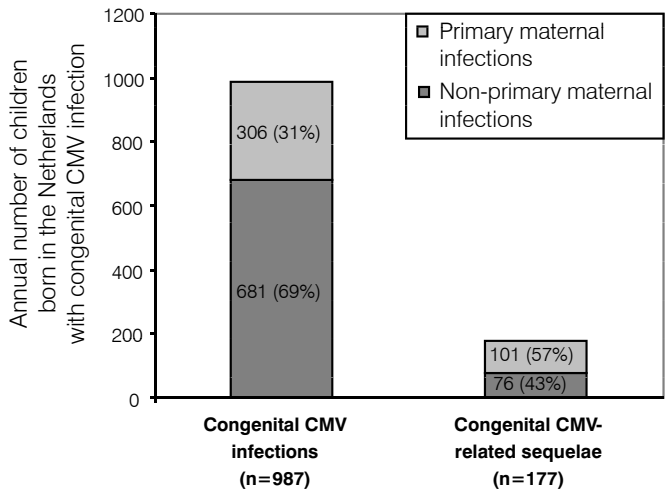

Figure 2 Annual number of children born in the Netherlands with congenital CMV infection and with CMV-related permanent neurological sequelae (at later ages) according to primary and non-primary maternal infection. Estimates were based on the population based predication model (*Chapter 4*) (50% CMV seroprevalence[17,21]) and the annual number of congenitally infected newborns based on the birth prevalence in the Netherlands (*Chapter 2*).

Similarly, additional calculations could be made when combining our model (*Chapter 4*) with seroprevalence data of subpopulations in the Netherlands found to be at higher risk of congenital CMV (*Chapter 3*). CMV seroprevalence data of subpopulations of Dutch and Turkish/Moroccan origin in the Netherlands were used (35% and 96% seroprevalence[17,21], respectively). Among mothers of Turkish/Moroccan origin, non-primary maternal infections were estimated to account for 91% of the congenital infections with long-term sequelae (Figure 3).

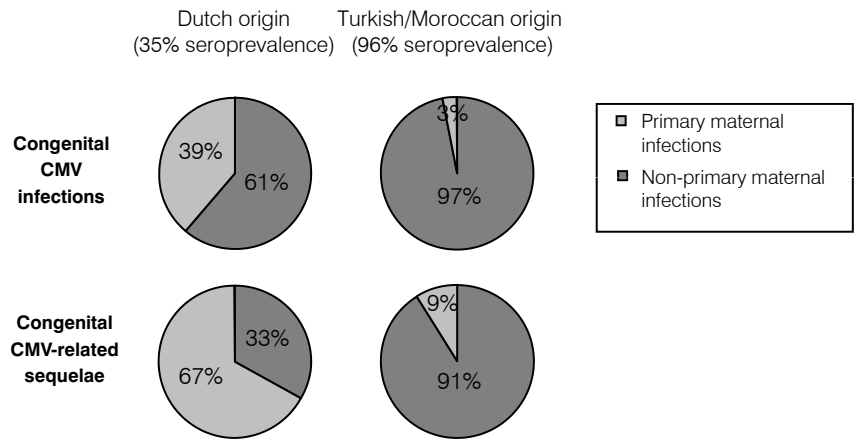

Figure 3 Estimated proportion of children with congenital CMV and CMV-related sequelae in the Netherlands among subpopulations of Dutch and of Turkish/Moroccan origin, according to non-primary and primary maternal infection (seroprevalence of 35% and 96% [17], respectively). Estimates were based on the population based prediction model (*Chapter 4*).

The apparent contradiction of maternal immunity as a risk factor for congenital CMV can be explained by the higher force of (re-)infection in highly seroprevalent (sub)populations.[22] Additionally, maternal re-activations may play a role. Awareness of the risk of seroimmune pregnant women of having a congenitally infected and neurologically affected newborn will have significant consequences for preventive strategies to reduce the disease burden of congenital CMV. Preventive measures such as hygiene counseling should not be limited to seronegative pregnant women. In that case, prenatal maternal serological screening will be futile as long as no adequate intervention option is available. Awareness of the fact that CMV immunity

is only partially protective for congenital infection raises questions on the ratio of re-infections with new strains versus reactivations of latent virus in seroimmune pregnant women. Passive and active immunization efforts should aim at provision of antibodies and vaccines for both seronegative and seropositive women, while, currently, an immunological correlate of full protection against congenital CMV infection and disease seems to be lacking. Recently, a CMV glycoprotein B vaccine has been shown to boost immunity in CMV seropositive women[23], however future studies are needed to determine the capacity of this vaccine to reduce congenital CMV infection and disease.

Recommendations for future studies

Practical data are desired to confirm our theoretical estimates, which were derived from a model, based on data from previous reports. A prospective study with follow-up of a large cohort of pregnant women would deliver data on the (re-)infection rate among pregnant women in the Netherlands. Preliminary data we obtained by means of an additional cross-sectional study in which sera from CMV seropositive pregnant women in the first trimester were assessed for CMV DNA, indicated that a very low proportion of these women was CMV viremic at the time of sampling (1/122, 0.8% of CMV IgG positive sera, data not shown). Furthermore, it would be interesting to distinguish re-infections with new strains from reactivations of latent virus in pregnant women. A recent serological study showed that re-infection with new strains was a major source of congenital infection, occurring in about 8% of seroimmune pregnancies in Brazil.[24] However, the proportion of maternal re-infections versus reactivations resulting in congenital infection is not known and further studies distinguishing CMV strains by means of serology and/or genome analysis (see *Chapter 8*) would be helpful. Immunization studies addressing the capacity of CMV vaccines to reduce maternal-to-fetal transmission rates among both seronegative and seroimmune women are needed. A search for correlates of protection of fetal infection and disease is essential.

Knowlegde of the responding obstetricians and gynecologists (in training) on congenital CMV infection was suboptimal (*Chapter 5*)

Implications of this finding

A digital questionnaire sent to interns, residents, senior doctors, general practitioners and medical researchers involved in mother and child care in the Netherlands, suggested that the responding physicians (in training) had suboptimal knowledge concerning congenital CMV. About half of the responding obstetricians and gynecologists (in training) were not aware of the fact that CMV is not transmitted by air and can be transmitted by kissing young children on the mouth and changing diapers (*Chapter 5*). Furthermore, only the minority of the respondents in pediatrics realized that newborns with congenital CMV may be asymptomatic at birth and that 1 out of 5 congenitally infected newborns will develop long-term sequelae. Our findings imply that congenital CMV infections may not be recognized by these physicians and therefore under-diagnosed with the risk of treatment delay or refrain. Furthermore, these physicians were not likely to be able to optimally advise on the risk of congenital CMV and how this risk may be reduced.

Recommendations for future work

Education of physicians on congenital CMV is expected to result in increased awareness, and awareness of physicians is essential for awareness of pregnant women and policy makers in health care. Increased knowledge and awareness of physicians and pregnant women is expected to improve recognition and care, to stimulate diagnostic investigations and audiological follow-up of infected newborns, and to enhance preventive measures. A two-fold reduction of the risk of seroconversion among pregnant women has been reported[20] after advising mainly three hygiene measures, also promoted by the CDC: 1. hand washing after diaper changes, 2. avoiding kissing young children on the mouth, 3. avoiding sharing utensils. A large study is needed to determine the effect of hygiene measures on the number of (prevented) congenital CMV infections. Overall, it is recommended that educational efforts are increased employing all possible methods to reach all groups involved.

OVERALL CONCLUSIONS OF PART I

Combining insights provided by the findings presented in part I of this thesis as well as from other available data, it can be concluded that the disease burden of congenital CMV in the Netherlands is considerable. Congenital CMV infection is the most frequent congenital disorder and appears to be the leading cause of non-genetic congenital hearing loss. Congenital CMV disease affects all subpopulations in the Netherlands, and seronegative as well as seropositive pregnant women are at risk of having a newborn with congenital CMV-related disabilities. The disease burden is striking when one realizes that a non-negligible part of the congenitally infected newborns with late-onset hearing loss is not detected in the newborn hearing screening, with delayed intervention for hearing loss as a consequence. Uncorrected prelingual hearing loss has profound negative effects on speech and language development, communication and learning, and affects the socio-economic status of the affected children and their families. Though an extensive analysis of the exact costs involved in congenital CMV disease is currently underway, lifetime costs of prelingual bilateral hearing loss, irrespective of etiology, are impressive (>700,000 euros per disabled individual[25,26,27,28]). Moreover, additional to the postnatal disease burden of congenital CMV addressed in this thesis, congenital CMV has been associated with intra-uterine fetal death[29,30,31,32], increasing its impact even further.

Taken together, it can be concluded that **congenital CMV infection can be labeled as an important public health problem**.

PART II POSTNATAL SCREENING TOOLS FOR CONGENITAL CMV

Sensitivity of CMV DNA detection in dried blood spots (DBS) varied widely, depending on the DNA extraction method used (*Chapter 6 and 7*)

IMPLICATIONS OF THIS FINDING

Sensitivity and applicability of several DNA extraction methods for high-throughput usage were assessed by means of *in vitro* experiments using Guthrie cards spotted with CMV positive blood. Significant differences were found between the extraction methods with respect to the sensitivity. Sensitivities ranged up to about 86% for Guthrie cards spotted with CMV DNA loads around the reported[33] median load of 3.4 $\log_{10}$ copies/ml for symptomatic and asymptomatic congenitally infected newborns. When considering the usage of DBS for universal newborn screening for congenital CMV infection, an assay which is sensitive, specific, and applicable for 96-well format testing, while using only a very small amount of dried blood, is required. When evaluating screening assays, the predictive values of screening test results are even more important than sensitivity and specificity. Considering a national congenital CMV birth prevalence of 0.54% (*Chapter 2*), a screening test with a sensitivity of 75% would still result in a negative predictive value as high as 99.8%. Furthermore, the demonstrated association between viral load and outcome[34,35,36,37] suggests that any cases missed would be those with the lowest viral loads and probably the lowest chance of developing severe permanent sequelae. Thus, the clinical sensitivity, based on the detection of children who will eventually develop sequelae, may well be acceptable.[38,39,40]

Recommendations for future studies

When considering universal newborn screening for congenital CMV infection, an assay which is sensitive, specific, and applicable for 96-well format testing is needed. In view of the existing route of the national metabolic screening program, DBS would be the most practical specimen of choice. Experience with DNA detection in newborn screening laboratories is accumulating, in particular in the postnatal screening for cystic fibrosis.[41] Therefore, it is interesting to further optimize DBS DNA extraction protocols, PCR techniques, testing algorithms, and test procedures. Large scale

prospective and retrospective studies have assessed several PCR-based assays for CMV detection in DBS and their results mainly correspond with our findings, reporting sensitivities of 71-100%.[42,43,44,45] In contrast, the widely commented[38,39] study by Boppana et al[46] reported a sensitivity as low as 34% of the specific DBS assay used to screen 20,448 newborns. Exploratory studies in which optimized CMV DBS assays are used for large-scale newborn screening are needed to address remaining analytic and logistic issues.

While further exploring DBS PCR assays, alternative assays with potential for sensitive and high-throughput detection of CMV may be explored. Recently, the use of dried saliva for screening for congenital CMV has been tested and found to be very sensitive. Table 1 summarizes clinical pilot studies on PCR-based newborn screening assays for congenital CMV infection reported to date. Future studies are likely to address the logistic feasibility of materials other than DBS in more detail. Potentially, logistic issues may be more challenging in countries where a large proportion of the children are born and sampled in their home environment.

Additionally, since current metabolic screening is mainly performed using mass spectrometric assays, it would be logistically advantageous to use mass spectrometric detection of CMV in DBS. While it may be difficult to detect relatively low amounts of CMV-specific proteins present in DBS, it would be worthwhile to explore mass spectrometric detection of CMV.

Table 1 Clinical pilot studies on newborn screening for congenital CMV infection reported in literature. Studies using PCR-based screening assays were included and predictive values were calculated.

Reference	Screening test (PCR-based) sample	Comparison (gold standard)	Sensitivity	Specificity	Birth prevalence	PPV	NPV
[43] (2011)	DBS[a]	Urine PCR	100% 96.9%	98.1% 99.0%	23.6% (64/271)[c]	94.1% 96.9%	100% 99.0%
[46] (2010)	DBS[b]	Saliva culture	34.4%	99.9%	0.5% (92/20,448)	91.7%	99.8%
[47] (2011)	Saliva Liquid Dried	Saliva culture	 100% 97.4%	 99.9% 99.9%	0.5% (177/34,989)	 91.4% 90.2%	 100% 99.9%
[48] (2006)	Saliva Urine	Urine and saliva culture[d]	85.7% 92.9%	100% 100%	1.5% (28/1923)	100% 100%	99.8% 99.9%
[49] (2010)	Urine Throat swab DBS	Urine and throat culture	100% 100% 50.0% (1/2)	100% 100% 100%	1.5% (2/137)[c]	100% 100% 100%	100% 100% 99.3%
[50] (2011)	Urine (pooled)	Urine culture	100%	100%	6.3% (10/160)[c]	100%	100%
[51] (2011)	Urine (on filter paper)	[e]	-[f]	-	0.3% (66/21,272)	94.0%	-
[52] (2009)	Urine and/or saliva	[e]	-	-	1.1% (87/8047)	100%	-
[53] (2008)	Urine (on filter paper)	[e]	-	-	0.4% (4/901)	100%	-
[54,55] (2003,2005)	Urine	[e]	-	-	0.7% (14/2000)	90.0%	-
[56] (2009)	Umbilical cord blood	[e]	-	-	0.2% (2/1010)	100%	-
[57] (2006)	Umbilical cord blood	-	-	-	0.5% (2/433)	-	-

[a] QIAamp DNA Blood Mini Kit (Qiagen) extraction using 1 whole DBS,
[b] Qiagen M48 robot (MagAtract) extraction using two 3-mm disks,
[c] Selected population of newborns,
[d] Comparison included a subset (n=100) of screening negative samples,
[e] Only positive screening test results were confirmed with urine and/or saliva culture,
[f] DBS sensitivity 75% (9/12)
PPV; positive predictive value, NPV; negative predictive value, DBS; dried blood spot

The multiplex real-time CMV glycoproteins B and H genotyping assays developed, were efficient, sensitive for detecting mixed infections in plasma, and applicable for usage on DBS (*Chapter 8*)

Implications of this finding

Detection of CMV DNA in DBS has been shown to be a challenge[44,45,42,46] due to the small amount of dried blood available (*Chapter 6*). In spite of this, using our genotype assay, a genotype could be assigned to approximately 75-80% of the CMV DNA positive DBS of congenitally infected newborns. Others have shown that genotyping of CMV has supported the discrimination of reactivation of latent virus from re-infection with new CMV strains in plasma from transplant patients, allowing a better definition of donor-to-recipient transmission patterns.[58] As described in *Chapter 4*, congenital CMV infections mainly result from recurrent maternal infections, comprising re-infections and possibly reactivations. Our genotyping tool might support the discrimination of maternal reactivation from re-infection, reveal mother-to-fetus transmission patterns and the clinical outcome of congenital infection after reactivation versus re-infection. Increased insight into transmission risks of latent and new strains may have significant implications for preventive and therapeutic strategies, including CMV vaccine research.

Recommendations for future studies

Future studies, analyzing a large number of newborns and their mothers should address the frequency of re-infections and reactivations, mother-to-fetus transmission patterns, and the potential role of congenital infections with multiple CMV genotypes. Additionally, it would be of interest to study the presence of genomic variants longitudinally within one human host. Recent genome-wide next-generation sequencing of CMV in urine of congenitally infected newborns suggested that the genomic intra-host variability of CMV (0.2% nucleotide diversity per sample) may be comparable to that of many RNA viruses.[59]

CMV was more frequently detected by real-time PCR than by viral culture of neonatal urine samples (Chapter 9)

Implications of this finding

A retrospective analysis of a large series of neonatal urine samples received for congenital CMV diagnostics, showed that CMV was more frequently detected by real-time PCR than by viral culture. False negative CMV urine culture results have been reported, both in experimental setting[60] and in clinical setting[61,62,63,64], and therefore seem the most likely explanation for our discrepant test results. Loss of viable CMV particles implicated in false negative culture results may be caused by transport at room temperature[65], and/or antiviral therapy. Our results, supported by analytical and clinical data on CMV DNA detection in neonatal urine, suggested enhanced sensitivity of recent PCR techniques when compared to viral culture. These combined findings provide considerable rationale to favor real-time CMV PCR as a gold standard in the diagnosis of congenital CMV infection.

Recommendations for future studies

Data from large-scale studies combining clinical data from newborns with diagnostic inhibition-controlled real-time CMV PCR procedures should address the differentiation between congenitally and postnatally acquired CMV infection, and should determine the exact time-frame for sampling of neonatal urine when using real-time PCR.

OVERALL CONCLUSIONS OF PART II: SCREENING TOOLS

Combining the findings presented in part II of this thesis with data from the literature, the overall conclusion would be that, now that several newborn screening tools for congenital CMV have been studied, PCR-based screening assays using DBS, saliva, and urine appear to be the most attractive tools currently available for newborn screening for congenital CMV. Whereas saliva and urine samples have the advantage of containing high viral loads[53] and potentially high test sensitivity, DBS have the major logistic advantage of being suitable for use in Guthrie card-based metabolic screening. The wide range of sensitivities of DBS PCR assays reported in (clinical pilot) studies including our own, provides the insight that sensitivity data of specific DBS PCR assays cannot be generalized. It appears that a sub-selection of DBS PCR assays with high DNA extraction capacity has the potential to achieve sensitivity and specificity levels approaching those of assays currently used in metabolic screening in the Netherlands (about 100% sensitivity and ≥99.97% specificity[66]). It must be noted that a lower analytical sensitivity may well be acceptable, since the previously demonstrated association between viral load and clinical outcome[34,35,36,37] suggests that any cases missed, would be those with the lowest viral loads and probably the lowest chance of developing severe permanent sequelae.

The use of dried urine samples collected on filter paper placed in diapers has been described as feasible for mass screening and should be explored.[51] When considering the use of (dried) urine samples for newborn screening for congenital CMV, a narrow time-frame for sampling must be taken into account, in order to differentiate congenital from postnatal infection.

Taken together, while currently available screening tools are being optimized and fine-tuned, **the technical stage appears to be set for newborn screening for congenital CMV**.

PART III PROS AND CONS OF NEWBORN SCREENING FOR CONGENITAL CMV

Despite previous appeals for preventive measures for congenital CMV infection[67,68], newborn screening for congenital CMV has only recently begun to be considered seriously. The potential for newborn screening for CMV lies in the identification of the large proportion of asymptomatic congenitally infected newborns at risk for developing late-onset hearing loss or other sequelae. There is growing support for two primary ideas: the benefit of hearing preservation in symptomatic newborns by means of **antiviral treatment**, and the benefit of early identification of late-onset hearing loss by means of extensive **audiological follow-up** in congenitally infected infants. It appears that, after many years of research, congenital CMV infection now satisfies most screening criteria of Wilson and Jungner.[40,69] Pros and cons for newborn screening for congenital CMV are addressed in detail in the following *Chapters 11* and *12*.

From these discussions and combining insights, as provided by the studies presented in this thesis as well as from other available data, the overall conclusion is that a large-scale study on the safety and efficacy of combined newborn screening and antiviral therapy is the necessary next step to take in the long-lasting fight against the damage caused by congenital CMV infections.

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Chapter 11

Implementing neonatal screening for congenital cytomegalovirus: addressing the deafness of policy makers

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Summary

Congenital cytomegalovirus (CMV) infection is an important public health problem with approximately 7 in 1,000 newborns infected and consequently at risk for hearing impairment. Newborn hearing screening will fail to detect this hearing impairment in approximately half of the cases because late onset hearing loss is frequent. Hearing impairment has profound impact on cognitive and social development of children and their families, determining most of the disease burden of congenital CMV infection. The potential value of newborn screening for congenital CMV is increasingly discussed. To date, many experts acknowledge the benefit of antiviral treatment in the prevention of hearing deterioration in newborns with neurological symptoms, and the benefit of early identification of late-onset hearing impairment by means of extensive audiological follow up of infected infants. These opinions imply that the potential of newborn screening for CMV would lie in the identification of the large proportion of asymptomatic congenitally infected newborns at risk for developing late-onset hearing loss. Experience with postnatal antiviral treatment of symptomatic newborns is encouraging, but has not been studied in asymptomatic congenitally infected newborns. A large-scale study on the safety and effectiveness of combined screening and antiviral therapy for congenital CMV infection is the necessary next step to take and should not be delayed.

Introduction

Despite the appeals for preventive measures for congenital cytomegalovirus (CMV) infection by Yow and Demmler in 1992 ''Congenital CMV disease—20 years is long enough'' [1] and the statement by Adler that, in 2005, ''there is considerable rationale for implementing neonatal screening now'' [2], newborn screening for congenital CMV has only recently begun to be explored. Indeed, in the last year, several original articles, editorials and reviews have been published on this subject.[3–11] In a recent review, Dollard et al.[8] showed that, after many years of research, congenital CMV infection now satisfies most screening criteria of Wilson and Jungner.[12] There is growing support[3,6,8,9,11] for two primary conceptions: the benefit of prevention of hearing deterioration in symptomatic newborns by means of antiviral treatment, and the benefit of early identification of late-onset hearing impairment by means of extensive audiological follow-up in congenitally infected infants. So now, after again almost 20 years, the stage appears to be set for neonatal screening.

The Wilson and Jungner criteria and newborn screening on congenital cmv

The Wilson and Jungner[12] criteria for newborn screening include the requirements that the disease has to be an important public health problem with a well understood history, that an early diagnosis can be made with a suitable screening test, and that the benefits outweigh the risks and costs of early intervention. The overall birth prevalence of congenital CMV is approximately 0.7%, and an estimated 18% of the congenitally infected newborns will develop permanent neurological sequelae.[13–16] Hence, congenital CMV is responsible for affecting approximately 126 in 100,000 newborns causing permanent neurologic sequelae, most prominently sensorineural hearing loss (SNHL), but also neurodevelopmental disabilities. In the 27 countries of the European Union (EU-27), every year 37,800 congenital CMV-infected babies are born, of which 6807 will eventually suffer from permanent sequelae (Figure 1). Among children with bilateral profound SNHL, the hearing disability is attributable to congenital CMV infection in one in five patients, making CMV the leading cause of non-genetic congenital hearing impairment.[14,17] Due to the frequently occurring late onset character of the hearing loss caused by congenital CMV, approximately half of the patients will pass the newborn hearing screening.[18]

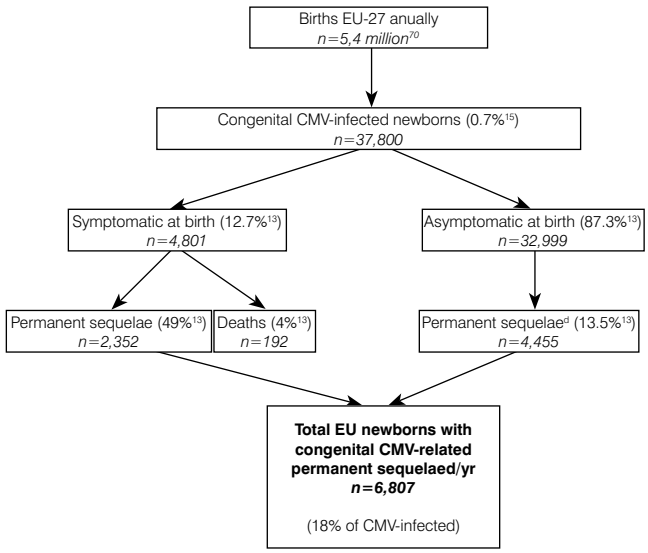

Figure 1 Congenital CMV disease burden in the EU-27.

Compared to several other diseases for which newborn screening has already been implemented, the prevalence of congenital CMV infections is notably high (Table 1). For example, sequelae caused by congenital CMV are more than 100 times more prevalent than homocystinuria, a partially untreatable disorder for which postnatal screening is standard care in most developed countries nowadays.[19]

One of the Wilson and Jungner criteria for newborn screening concerns the availability of an acceptable screening test, suitable for diagnosis in an early stage of the disease. Newborn screening for congenital CMV infection would indeed identify newborns at risk for developing late-onset hearing loss at an early stage. Dollard et al.[8] have reviewed several laboratory aspects of newborn screening for congenital CMV. In view of the existing routes of national metabolic screening programmes, dried blood spots (DBS) would be the most practical specimen of choice. CMV DNA detection in DBS is technically feasible and has become routine practice in an increasing number of clinical microbiological laboratories.[20] Experience with DNA detection in newborn screening laboratories is accumulating, in particular in the postnatal screening for cystic fibrosis.[19] Specificity of CMV PCR assays on DBS has been reported to range between 99.3% and 100%[21–23], with a specificity approaching 100% as a prerequisite for an acceptable positive predictive value.

Table 1 Several disorders included in newborn screening in EU countries with their prevalences, clinical outcome if untreated and efficacy of early intervention.

Several disorders included in newborn screening	Rate per 100,000 newborns (EU)	Clinical outcome if untreated	Efficacy of early intervention	References
Congenital CMV (proposed)	700	From asymptomatic to severe neurological damage (18%, n =126/700)	Partially treatable: prevention of deterioration of hearing with ganciclovir	13–15
Congenital hypothyroidism	45	From asymptomatic to severe mental retardation	Treatable: thyroxine prevents mental retardation	71
Cystic fibrosis	30	COPD, pancreas—and liver fibrosis	Mainly untreatable: limited to improved feeding status and genetic counselling of parents	72
Homocystinuria	1	From mild to severe, including thromboembolism, mental retardation and ectopia lentis	Partially treatable: vitamin B_6 responsive and non-responsive form	73

EU, European Union; CMV, cytomegalovirus.

Additional confirmatory testing of newborns with CMV positive DBS, using urine sampled within the first 2–3 weeks after birth, the current gold standard, would increase specificity to 100% (positive predictive value of 100%).

The issue has been raised whether the sensitivity of DBS testing for CMV DNA is adequate for screening purposes.[4,5,7,9] Previously reported analytical and clinical sensitivities of CMV DNA detection using DBS vary within a wide range from 34% by Boppana et al.[4] up to 100%.[10,20,23–31] The wide range in reported sensitivities can be explained by the population of newborns tested (proportion of asymptomatic and symptomatic cases), and the testing method used. A small number of prospective studies have tested sensitivity of CMV DNA detection in DBS in a large population of unselected newborns in comparison with the gold standard, i.e. urine CMV culture or PCR at 2–3 weeks after birth. Soetens et al.[30] reported sensitivities up to 83% testing DBS from 55 CMV-infected newborns detected with a large urine screening program in an unselected population. Yamamoto et al.[32] reported a sensitivity of 71% testing 332 DBS from urine screened unselected newborns of whom seven with congenital CMV infection. Johansson et al.[28] described a sensitivity of 81% testing DBS from 16 congenitally infected newborns identified by means of urine screening. In contrast, the annotated[5,7] study by Boppana et al.[4] reported a sensitivity as low as 34% of the DBS assay used to screen 20,448 newborns compared to saliva testing. However, the most recent report on sensitivity of DBS testing by Kharrazi et al.,[10] screening 3972 newborns using DBS, measured a prevalence similar to reports using established methods for diagnosing congenital CMV infection, suggesting an adequate sensitivity. The major factor responsible for these considerable differences in reported sensitivities of DBS assays, even when assessing an unselected population of newborns in comparison with the gold standard, is the testing method used.[5,7] Widely different DBS test protocols have been used, including variations in DNA extraction methods. It has been demonstrated that these differences in DBS test protocols result in major divergences in sensitivity.[25] Thus, sensitivity results obtained using one specific DBS testing protocol cannot be generalised to other DBS testing methods. Optimising DNA extraction protocols, PCRs, and testing algorithms, e.g. by means of performing independent triplicate testing, have been shown to increase analytical sensitivity significantly.[25,27,30] Recently, Gohring et al.[27] calculated a detection limit as low as 200 copies CMV-DNA per millilitre using a highly sensitive protocol. More important than the sensitivity when evaluating screening assays is the negative predictive value. Considering an international birth prevalence of 0.64%, a screening test with a sensitivity of 75% would still result in a negative predictive value as high

as 99.84%. It appears that a perceived lack of analytical sensitivity need not be a diagnostic limitation. Furthermore, the previously demonstrated association between viral load and clinical outcome[33–36] suggests that any cases missed would be those with the lowest viral loads and probably the lowest chance of developing severe permanent sequelae. Thus, as Dollard et al. also mentioned, the clinical sensitivity, based on the detection of children that will eventually develop sequelae, may well be acceptable.[5,7,8] Obviously, high-throughput testing should be optimised before implementing universal neonatal screening.[25] It appears that with optimal quality assurance, a high specificity and a sufficient clinical sensitivity can be achieved, enabling exploratory regional trials for large-scale newborn screening.

Postnatal interventional options

As described by Wilson and Jungner[12], the benefits of newborn screening and intervention should outweigh potential physical and psychological disadvantages. The major benefit of newborn screening for congenital CMV would be early identification of newborns at risk for developing late-onset hearing loss. The current universal newborn hearing screening fails to detect approximately half of all SNHL caused by congenital CMV infection[18] and presently, the median age of detection of hearing impairment caused by congenital CMV infection is approximately 2 years.[37] Hearing impairment in the first 3 years of life has detrimental effects on speech and language development.[38,39] Correction of hearing impairment with hearing aids or cochlear implantation is most effective prior to the age of 6 months.[38,39] At that early stage, correction of hearing can result in communicative and linguistic skills very similar to those of their normally hearing peers.[38,39] Newborn screening for congenital CMV would enable the identification of the 0.7% of newborns at risk for developing hearing impairment due to congenital CMV, potentially followed by intensive follow-up of audiological performance in this selected group. Audiological follow-up of up of all newborns without screening for congenital CMV is not an attractive alternative due the enormous numbers of newborns involved with the logistic, psychological, and financial consequences attached.

The ultimate benefit of newborn screening would come from the prevention of both early and late-onset hearing deterioration. Any reduction in the number of children with severe to profound hearing loss will have great impact on the burden of disease, influencing both the quality of life of the patients and the economic burden of disease. One randomised controlled trial with intravenous ganciclovir therapy for 6 weeks significantly reduced hearing deterioration in a selected group of symptomatic

newborns with congenital CMV infection involving the central nervous system (microcephaly, intracranial calcifications, abnormal CSF, chorioretinitis, and/or hearing deficits).[40] Sixty-eight % of the untreated infants in the trial had hearing deterioration at the age of 1 year versus 21% of the ganciclovir-treated infants, resulting in an efficacy of 69%. Additionally, ganciclovir had a beneficial effect on the neurological development (personal/social and motor development) of these infants.[41] Although this study had some major drawbacks, such as the high number of cases lost to follow-up and the lack of the usage of a placebo in the untreated group, these results have led to the general opinion that this subgroup of congenitally infected children with neurological symptoms should be treated with at least 6 weeks of (val)ganciclovir. Subsequent trials with this particular group of symptomatic children have actually not included a placebo-group (www.clinicaltrials.gov, accessed December 2010).

Despite the encouraging results in symptomatic children, the benefit of antiviral therapy in asymptomatic newborns with congenital CMV infection has not yet been proven to date. For this reason, this intervention is not included in current guidelines.[42,43] To our knowledge, only one randomised controlled trial with asymptomatic congenitally infected newborns without hearing loss has been reported studying the effect of 3 weeks intravenous ganciclovir on hearing.[44] During 4 to 10 years of follow-up, none of 10 treated infants developed hearing loss, compared with two out of eight untreated infants. Unfortunately, this study lacked statistical power to draw firm conclusions about the efficacy of the antiviral treatment in this group. In addition, Yilmaz-Ciftdogan et al.[45] reported the improvement of bilateral hearing impairment in an otherwise asymptomatic congenitally infected newborn treated with intravenous ganciclovir for 1 week followed by oral valganciclovir for five additional weeks.

Valganciclovir, which can be administered as a convenient oral solution, is now considered an adequate and practical substitute of the previously applied intravenous formulation of ganciclovir.[46–48] In many other (pediatric) settings, both ganciclovir and valganciclovir have increasingly been tested and used, also for prolonged periods. (Val)ganciclovir has side-effects, with neutropenia being the most common one. A moderate to severe neutropenia is seen in approximately one out of five untreated newborns with congenital CMV infection and in an additional two out of five ganciclovir treated newborns.[40,45] This neutropenia is transient and reversible within a few days upon dose reduction or discontinuation of the drug. Human data on the potential long-term side effects of the active substance of valganciclovir, ganciclovir are lacking. The only data come from a small number of animal studies in which carcinogenic and aspermatogenec effects have been observed.[49,50] Ganciclovir was

carcinogenic in mice at doses that produced concentrations of 0.1 and 1.4 times the mean drug exposure in humans.[49] Additionally, ganciclovir decreased fertility in mice at concentrations comparable to human usage, whereas embryotoxicity in pregnant rabbits and mice have only been observed at twice the drug concentrations obtained in humans.[49] It is unclear to what extent these limited data can be extrapolated to humans. Future data from a lifetime of human usage will position these long-term side effects in the proper perspective. To date, no reports have been published on documented or suspected carcinogenic or teratogenic effects due to (val)ganciclovir, despite its extended usage in adults and its growing usage in the paediatric publication since the first publication on ganciclovir in 1982.[51]

Though randomised controlled-trials should provide further evidence, there are data that support the hypothesis that antiviral therapy has a role in preventing hearing loss in asymptomatic newborns. Several findings suggest that ongoing viral replication is responsible for CMV-associated SNHL. First, CMV-induced labyrinthitis has been demonstrated in human cases and animal model studies.[52–56] Viral DNA has indeed been detected in the perilymph of children with congenital CMV infection at ages ranging from one to 19 years.[57–60] Finally, indirect evidence of a viral replication-associated pathogenesis can be found in the previously published relationship between CMV viral load in the newborn and the occurrence of SNHL[33–36,61–63], the late-onset character of the hearing loss[18,64] and the beneficial effect of antiviral treatment in reducing the development or deterioration of SNHL.[40,41] On the other hand it has been shown that treatment with intravenous ganciclovir or oral valganciclovir will reduce CMV viral load in a predictable pattern as shown by Emery et al.[65] Since the majority of children with congenital CMV infection are asymptomatic at birth, studies are required to define their baseline viral load and determine if this can be efficiently reduced to an undetectable and safe level.

To initiate postnatal antiviral treatment in initial asymptomatic children is a difficult decision, due to the fact that about 82% of the children with congenital CMV infection will not develop any sequelae[13] but will be treated with an antiviral drug with potential side-effects. However, the potential lifelong benefit for those that will have severe hearing loss and possibly neurodevelopmental delay has to be balanced against this disadvantage of a preemptive strategy. To achieve a benefit ratio of 10 newborns needed to treat to obtain benefit for one child, the efficacy of antiviral treatment of approximately 70% is needed, based on the natural history of development of hearing loss as described by Fowler et al.[64] To date, no data are available on the efficacy of antiviral therapy in initially asymptomatic newborns, and therefore, a well-considered

appraisal cannot be made at this moment. Considering that potential harm would be mild and temporary whereas potential benefit would be substantial and permanent, the preventive measure of combined neonatal screening and antiviral treatment is certainly worth to be studied in a randomised controlled trial. Ongoing research will lead to insight into the optimal treatment strategy and duration and should reveal both viral and host factors involved in clinical outcome, potentially leading to a defined risk group that would benefit most from antiviral treatment.

Cost-effectiveness

No data are available published on the cost-effectiveness of newborn screening for congenital CMV infection followed by intervention as compared to refraining from any screening or intervention. However, reliable data exist on the disease burden due to congenital CMV infection and the number of children with permanent sequelae. On the EU-27 scale, implementing a congenital CMV newborn screening program would detect approximately 37,800 newborns (Figure 1) with congenital CMV. The current lack of efficacy data on early antiviral treatment is hampering a detailed cost-effectiveness analysis at this moment. However, data on lifetime costs of hearing impairment, irrespective of the etiology, are available.[66–69]

Lifetime costs include assistive devices, medical costs, special education and lost productivity, and (in 2007) were estimated to be over € 700,000 per person with prelingual bilateral hearing loss.[66–69] The costs of prevention of hearing deterioration of partially unilateral and bilateral hearing impairment as caused by congenital CMV (cost-of illness) are not exactly reported and differentiated. However, it would be worthwhile to weigh the costs and benefits of newborn screening followed by intervention when insight in efficacy of treat-ment of initially asymptomatic newborns is expanded. Given the enormous costs of hearing impairment contracted in early childhood, there is potential for substantial cost reduction.

Conclusion

Now that an increasing number of the Wilson and Jungner criteria for newborn screening have been met, a large-scale study on the effectiveness of newborn screening for congenital CMV infection is the necessary next step to take. Further delay should be considered undesirable and unjustifiable. Policy makers in healthcare should take action now, as the infected infants deserve the benefit of the doubt.

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Chapter 12

Screening newborns for congenital cytomegalovirus infection

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To the Editor:

Dr Boppana and colleagues[1] concluded that dried blood spot (DBS) real-time PCR assays are not suitable for screening newborns for congenital CMV infection due to their insufficient sensitivity.

[*Boppana et al. published in JAMA (2010) results of a multicenter study in which 20 448 newborns were screened for congenital CMV infection by means of rapid culture of saliva specimens and 2 different in-house DBS real-time PCR assays. Congenital infection was confirmed by means of rapid culture of saliva or urine in 92 infants. Sensitivity and specificity of these PCR assays were at most 34.4% and 99.9%, respectively. Negative and positive predictive values were 99.8% and 91.7%, respectively. The authors stated that, DBS real-time PCR assays have limited value for screening newborns for congenital CMV infection because of insufficient sensitivity.*]

We believe that this is a premature conclusion, based on a number of considerations that were not sufficiently discussed in this article.

First, the sensitivity of DBS testing is highly variable, largely depending on the nucleic acid extraction methodology used,[2] so conclusions cannot be generalized. It appears that this problem can be reduced by using optimized techniques that differ from those applied in the study by Boppana et al [*Qiagen M48 robot (MagAtract) extraction using two 3-mm disks of dried blood*].[1] In addition, performing independent triplicate testing to increase sensitivity has been advocated,[2] an approach not used in this study.

Second, it should be clear what the clinical relevance is of the cases that were missed. These cases will likely involve the samples with the lowest or even absent viral loads, and there is evidence that such cases are associated with lower risks of late-onset sequelae, including hearing loss.[3] Sensitivity should be judged by patients in whom hearing loss is eventually caused by CMV. The intended follow-up of the infants with congenital CMV infection in this study will reveal the clinical outcome, and these data should be awaited before discarding the screening test that was used.

Third, we are concerned about the possible inclusion of very common but generally harmless postnatal CMV infections. Oropharyngeal contamination during vaginal delivery might cause positive saliva samples soon after birth, as has been shown for herpes simplex virus.[4] Sampling in this study was mainly performed on the day of birth. Confirmation of the presumed congenital infections was carried out at a mean age of more than 6 weeks, although it is commonly accepted that only CMV infections diagnosed within the first 2 or 3 weeks can be considered proof of congenital CMV infection.[5] If postnatally infected neonates were indeed included, this would falsely suggest a lower sensitivity of DBS testing.

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Samenvatting voor de niet medisch onderlegde lezer

AANGEBOREN CYTOMEGALOVIRUS INFECTIE: ZIEKTELAST en SCREENINGSTESTEN

-Op weg naar screening van pasgeborenen-

Algemene introductie

Cytomegalovirus

Virussen bestaan slechts uit een hoeveelheid erfelijk materiaal ingesloten in een omhulsel van eiwit met eventueel vet en hebben daarom een gastheercel nodig om zich te vermenigvuldigen, in tegenstelling tot bacteriën. Het cytomegalovirus (CMV) behoort tot de Herpesvirus familie, zoals ook het Epstein-Barr virus (de verwekker van de ziekte van Pfeiffer), het herpes simplex virus (de verwekker van de koortslip), en het varicella-zoster virus (de verwekker van waterpokken en gordelroos). CMV komt wereldwijd veel voor en wordt van persoon tot persoon overgedragen via direct contact met lichaamsvloeistoffen waarin het virus wordt uitgescheiden. Met name urine en speeksel van jonge kinderen kunnen grote hoeveelheden CMV bevatten en spelen daarom een grote rol bij overdracht van het virus. CMV wordt niet door hoesten overgedragen. Infectie met CMV bij gezonde volwassenen en kinderen verloopt meestal zonder klachten. In sommige gevallen ontstaat er echter een ziektebeeld vergelijkbaar met Pfeiffer, bestaand uit keelpijn, koorts, en vergrote klieren in de hals. Na infectie blijft CMV (net als alle virussen van de Herpesvirus familie) levenslang in het lichaam aanwezig, maar meestal zonder dat het klachten veroorzaakt. Bij personen met een slechte afweer (patiënten met HIV, kanker, of na een transplantatie) kan CMV zich opnieuw gaan vermenigvuldigen en schade veroorzaken aan het darmstelsel, de lever, de longen, en/of de ogen.

In Nederland is ongeveer de helft van de volwassenen ooit geïnfecteerd geraakt met CMV en heeft als gevolg daarvan afweer tegen het virus. Onder bepaalde bevolkingsgroepen buiten en binnen Nederland heerst er meer CMV, met name als gevolg van frequenter contact met jonge kinderen (bijvoorbeeld in (sub)culturen met grote gezinnen, crèches, en minder hygiënische omstandigheden).

Aangeboren (congenitale) infectie met CMV

Overdracht van CMV van moeder naar ongeboren kind vindt plaats via de placenta. In tegenstelling tot bijvoorbeeld rodehond kan CMV ook worden overgedragen op het ongeboren kind als de zwangere al vóór de zwangerschap geïnfecteerd is geweest met CMV en afweer heeft tegen het virus. Ongeveer 1 op de 10 pasgeborenen met een in de baarmoeder verworven (ofwel congenitale) infectie met CMV heeft bij geboorte geelzucht, bloedafwijkingen, vergrote lever, en/of schade aan het zenuwstelsel (onderontwikkelde hersenen, slechthorendheid en/of oogontsteking). Ongeveer de helft van de pasgeborenen met afwijkingen bij geboorte zal levenslang beperkingen

ondervinden. Van de congenitaal geïnfecteerde pasgeborenen zonder afwijkingen bij geboorte, zal ongeveer 1 op de 10 in de eerste levensjaren alsnog afwijkingen krijgen (geduid als 'late-onset'). Het meest voorkomend is slechthorendheid, daarnaast kan het mentale achterstand, vertraagde ontwikkeling, slecht zien, en/of epileptische aanvallen betreffen. Pasgeborenen met een moeder die reeds voor de zwangerschap afweer tegen CMV had, zijn meestal minder ernstig ziek. Een congenitale CMV infectie kan worden aangetoond met behulp van urine, speeksel en/of de hielprikkaart van de pasgeborene. In Nederland wordt bij alle pasgeborenen een paar druppels bloed uit de hiel afgenomen en opgevangen op een kaart (de hielprikkaart) voor routine screening op 18 erfelijke (stofwisselings)ziekten. De hielprikkaart wordt niet routinematig getest op CMV. Een pasgeborene met afwijkingen veroorzaakt door congenitale CMV kan worden behandeld met een antiviraal medicijn. Recent onderzoek laat zien dat deze behandeling tevens op de lange termijn slechthorendheid kan voorkomen. Het is nog niet onderzocht of antivirale behandeling ook slechthorendheid kan voorkomen bij congenitaal geïnfecteerde pasgeborenen zonder afwijkingen bij geboorte.

Dit proefschrift

Het doel van dit proefschrift was om zowel de noodzaak als de haalbaarheid van screening van pasgeborenen op congenitale CMV in Nederland te onderzoeken. Hiervoor werd respectievelijk de ziektelast van congenitale CMV in Nederland in kaart gebracht en een groot aantal beschikbare testen voor detectie van CMV bij pasgeborenen onderzocht.

Ziektelast

In dit proefschrift werd de ziektelast van congenitale CMV in Nederland bestudeerd op verschillende manieren. Het vóórkomen van congenitale CMV infecties in Nederland werd onderzocht met behulp van een grote steekproef van hielprikkaarten, welke getest werden op aanwezigheid van CMV. In totaal **5 op de 1000 pasgeborenen** in Nederland bleek congenitaal geïnfecteerd te zijn met CMV. Dit betekent dat er in Nederland jaarlijks ongeveer **1000** kinderen geboren worden met congenitale CMV infectie, waarvan ongeveer **180** kinderen lange termijn gevolgen zullen ondervinden, met name slechthorendheid. Een aanvullende analyse van risicofactoren toonde aan dat congenitale CMV infecties vaker voorkomen in regio's met meer jonge kinderen en in regio's met meer immigranten.

De ziektelast van congenitale CMV in Nederland werd tevens bestudeerd door de proportie congenitale CMV infecties onder slechthorende kinderen te bepalen (met behulp van hielprikkaarten). In totaal 8% van de slechthorende kleuters, en maar liefst **1 op de 5 dove kleuters** in Nederland bleek congenitaal geïnfecteerd te zijn met CMV. De slechthorendheid op kleuterleeftijd was bij een belangrijk deel (25%) van de kinderen met CMV niet geconstateerd bij de routine gehoorscreening van pasgeborenen.
Tevens werd een schatting gemaakt van het aandeel congenitale CMV infecties onder zwangere vrouwen met reeds voor de zwangerschap afweer tegen CMV, met behulp van een theoretisch model. Paradoxaal genoeg bleek het aandeel congenitaal geïnfecteerde pasgeborenen onder moeders met afweer tegen CMV groter dan het aandeel onder moeders zonder afweer tegen CMV. Waarschijnlijk hebben moeders die al ooit een infectie met CMV hebben opgelopen een groter risico om opnieuw geïnfecteerd te worden door CMV (re-infectie), waarbij leefomstandigheden een rol spelen.
Geconcludeerd kan worden dat de ziektelast van congenitale CMV infecties in Nederland aanzienlijk is. Congenitale CMV is de belangrijkste oorzaak van niet-genetisch bepaald, aangeboren gehoorverlies. Congenitale CMV infecties komen frequenter voor dan het syndroom van Down, open ruggetje, en diverse erfelijke (stofwisselings)ziekten waarop hedendaags gescreend wordt bij zwangeren dan wel pasgeborenen. De restverschijnselen van congenitale CMV infectie zoals slechthorendheid en mentale retardatie zijn ingrijpend en hebben levenslang negatieve gevolgen voor taalontwikkeling, spraakontwikkeling, communicatie en leervermogen, en beïnvloeden de sociale en economische status van de getroffen kinderen en hun families. Kortom, congenitale CMV infecties zijn een belangrijk maatschappelijk gezondheidsprobleem.

Screeningstest

Diverse potentiële screeningstesten voor het aantonen van congenitale CMV bij pasgeborenen met behulp van de hielprikkaart werden bestudeerd in dit proefschrift. De capaciteit van deze testen varieerde aanzienlijk afhankelijk van de testmethode. Optimalisatie van de techniek resulteerde in verbeterde capaciteit waarbij ongeveer 80% van alle pasgeborenen met congenitale CMV zouden kunnen worden opgespoord. Andere onderzoekers laten zien dat met behulp van urine en speeksel mogelijk nog meer gevallen opgespoord zouden kunnen worden. Echter, testen gebaseerd op de hielprikkaart hebben als voordeel dat gebruik gemaakt zou kunnen worden van de huidige logistiek van de pasgeborenen screening op erfelijke (stofwisselings)ziekten.

Samengevat lijkt screening van pasgeborenen op congenitale CMV technisch steeds meer haalbaar nu potentiële screeningstesten worden geoptimaliseerd.

Discussie: screening van pasgeborenen

De optie van screening van pasgeborenen op congenitale CMV wordt in toenemende mate serieus overwogen in de medische wereld. De toegevoegde waarde zou liggen in de identificatie van geïnfecteerde pasgeborenen die niet anderszins (klinische presentatie, gehoorscreening) opgespoord zouden worden, maar wel risico lopen op gehoorschade en andere restverschijnselen in de eerste levensjaren. In de medische wereld wordt het belang ingezien van zowel het beschermend effect van vroege antivirale behandeling op het gehoor van geïnfecteerde pasgeborenen met afwijkingen, als het voordeel van vroege identificatie (en correctie) van gehoorverlies d.m.v. frequente controle van het gehoor van geïnfecteerde kinderen. Congenitale CMV lijkt momenteel te voldoen aan de meeste criteria die zijn opgesteld voor screening. Terwijl de ervaring met antivirale behandeling van geïnfecteerde pasgeborenen bemoedigend is, is het nut van deze behandeling bij geïnfecteerde pasgeborenen zonder verschijnselen nog niet bestudeerd.

Daarom is een grootschalige studie naar de veiligheid en effectiviteit van screening en behandeling van pasgeborenen de eerstvolgende te nemen stap in de langdurige strijd tegen de schade veroorzaakt door congenitale CMV infecties.

Curriculum Vitae

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