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A Pilot Study Using Residual Newborn Dried Blood Spots to Assess the Potential Role of Cytomegalovirus and *Toxoplasma gondii* in the Etiology of Congenital Hydrocephalus

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Abstract

BACKGROUND—Congenital hydrocephalus is a condition characterized by accumulation of cerebrospinal fluid in the ventricles of the brain. Prenatal infections are risk factors for some birth defects. This pilot study investigated whether residual dried blood spots (DBS) could be used to assess infections as risk factors for birth defects by examining the associations between prenatal infection with *Toxoplasma gondii* (*T. gondii*) or cytomegalovirus (CMV) with congenital hydrocephalus.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention or of the California Department of Public Health.

METHODS—Case-infants with hydrocephalus ($N = 410$) were identified among live-born infants using birth defects surveillance systems in California, North Carolina, and Texas. Control-infants without birth defects were randomly selected from the same geographic areas and time periods as case-infants ($N = 448$). We tested residual DBS from case- and control-infants for *T. gondii* immunoglobulin M and CMV DNA. When possible, we calculated crude odds ratios (cORs) and confidence intervals (CIs).

RESULTS—Evidence for prenatal *T. gondii* infection was more common among case-infants (1.2%) than control-infants (0%; $p = 0.11$), and evidence for prenatal CMV infection was higher among case-infants (1.5%) than control-infants (0.7%; cOR: 2.3; 95% CI: 0.48, 13.99).

CONCLUSIONS—Prenatal infections with *T. gondii* and CMV occurred more often among infants with congenital hydrocephalus than control-infants, although differences were not statistically significant. This pilot study highlighted some challenges in using DBS to examine associations between certain infections and birth defects, particularly related to reduced sensitivity and specimen storage conditions. Further study with increased numbers of specimens and higher quality specimens should be considered to understand better the contribution of these infections to the occurrence of congenital hydrocephalus.

Keywords

newborn dried blood spots; hydrocephalus; congenital infections; cytomegalovirus; *Toxoplasma gondii*

INTRODUCTION

Maternal infection during pregnancy can adversely affect pregnancy outcomes. Known teratogenic pathogens include rubella, cytomegalovirus (CMV), lymphocytic choriomeningitis virus, and *Toxoplasma gondii* (*T. gondii*), the cause of toxoplasmosis (Bale, 2002, 2009; Jamieson et al., 2006; Ornoy and Diav-Citrin, 2006). Because infections with some agents (e.g., CMV and *T. gondii*) can be asymptomatic or lead to nonspecific symptoms, studies relying on maternal report of infection are unlikely to discern their potentially etiologic role (Bale, 2002; Jamieson et al., 2006; Rasmussen et al., 2007; Wright et al., 1997). To improve understanding of the association between maternal infections during pregnancy and the occurrence of congenital anomalies, a different approach is required.

Congenital hydrocephalus is a condition, present at birth, characterized by the accumulation of cerebrospinal fluid in the ventricles of the brain (McAllister, 2012). Without surgical intervention to relieve the pressure caused by excess fluid, major damage to the brain tissue occurs in most cases. Congenital hydrocephalus is generally diagnosed prenatally or at birth, but it can also remain undiagnosed until later in life (Van Landingham et al., 2009). A pooled prevalence estimate from birth defects surveillance data collected in Arkansas, California, Georgia, Iowa, Massachusetts, New York, North Carolina, Texas, and Utah indicates that congenital hydrocephalus occurs in ~6.73 per 10,000 live births (Honein et al., 2012). While some cases of hydrocephalus are due to genetic causes, often no etiology is identified. *T. gondii* and CMV have the ability to infect the developing fetus and have been

identified as a rare cause of hydrocephalus, based primarily on case reports (James, 1992; Azam et al., 2001; Bale, 2002; Lipitz et al., 2002; Villena et al., 2010). Limited information is available to understand the magnitude of the contribution these infections make to the occurrence of congenital hydrocephalus on a population level.

Newborn residual dried blood spots (DBS) are stored either short- or long-term by many newborn screening programs after their initial screening use (Olney et al., 2006; Therrell et al., 2011; Therrell and Hannon, 2012) and represent an underutilized population-based resource for retrospective studies of *in utero* exposures to prenatal infections and other maternal exposures (Henderson et al., 1997; Snijdewind et al., 2012). This study was designed as a pilot to investigate the utility of DBS to assess infections during pregnancy as risk factors for hydrocephalus.

MATERIALS AND METHODS

Case Identification

Case-infants with hydrocephalus ($N = 410$) were retrospectively identified among live-born infants using population-based birth defects surveillance systems in CA, NC, and TX. The CA Birth Defects Monitoring Program reported cases born between 1995 and 2003 to mothers who were residents of 13 CA counties (Los Angeles, Orange, San Francisco, Santa Clara, San Diego, Fresno, Kern, Kings, Madera, Merced, San Joaquin, Stanislaus, and Tulare). The NC Birth Defects Monitoring Program reported cases born throughout the state between 2003 and 2005, and the TX Birth Defects Registry reported cases born throughout the state between 2003 and 2004. All identified cases were reviewed by a clinical geneticist with birth defects expertise. Cases of hydrocephalus due to a structural brain lesion or due to a known genetic cause or an intraventricular hemorrhage were excluded. Infants without birth defects were randomly selected from the same geographic area and time period as case-infants to serve as controls ($N = 448$). The institutional review boards at each state and the Centers for Disease Control and Prevention (CDC) approved this study.

Specimen Testing

A single residual DBS of ~1.3 cm in diameter was obtained from storage in CA, NC, and TX for each case-and control-infant. Specimens were transported under ambient conditions to the CDC. Upon receipt, all samples were stored at -20°C before analysis. Before transport to CDC, the CA DBS samples were stored in ideal conditions (-20°C , with desiccant, and exposed to, $<30\%$ humidity; Mei et al., 2011). In contrast, the NC and TX DBS samples were stored under ambient temperatures without desiccant; TX samples were stored in a dehumidified laboratory. Before being sent to CDC for analysis, specimens were stripped of all personal identifiers and labeled with a unique ID number (Mei et al., 2011). Individual level information was only maintained for four variables: case/control status, maternal state residence at delivery (CA, NC, and TX), maternal race/ethnicity (non-Hispanic white, non-Hispanic Black, Hispanic, and other), and maternal age (<30 years, 30 years). To ensure quality control, known *T. gondii*-positive ($n = 13$) and negative ($n = 10$) DBS samples, and known CMV-positive ($n = 13$) and negative ($n = 10$) DBS samples were

added into the study sample. Technicians performing the assays were blinded to case/control and quality control sample status.

Presence of *T. gondii* immunoglobulin M (IgM) was tested using the Neonatal Toxoplasma-screen AutoDelfia[®] kit (Kankaanpää et al.; PerkinElmer Life and Analytical Sciences, Turku, Finland) using a manual protocol (Zhou et al., 2005). Briefly, 3.2 mm disks were punched from the DBS and placed in Nunc cryotubes[™] (Thermo Fisher Scientific, Rochester, NY). Specimens were eluted overnight in the assay buffer at 4 °C. The cutoff for the presence of *T. gondii* IgM was 20 IU/ml (Zhou et al., 2005; Mei et al., 2011). For samples above the cutoff point, the infant was presumed infected with *T. gondii in utero*. While all specimens were tested for *T. gondii*, residual DBS samples from NC and TX were stored at ambient temperature and humidity, allowing IgM antibodies in DBS to degrade, prohibiting reliable *T. gondii* IgM elution. Therefore, *T. gondii* IgM results presented are limited to those from CA ($n = 515$; Mei et al., 2011).

CMV DNA was extracted from DBS using a modified thermal shock method (Shibata et al., 1994; Barbi et al., 1996). Three 3.2 mm punches were placed in a 2 ml microfuge tube with 60 μ l of Glutamine-free Minimum Essential Medium and soaked for 2 hr at room temperature, then incubated at 55 °C for 1 hr, then 100 °C for 7 min, and then transferred to ice for rapid cooling. The tubes were stored at -80 °C until they were used for polymerase chain reaction (PCR) analysis. Presence of CMV DNA was established using Taqman-based PCR targeting the CMV glycoprotein B region (Boppana et al., 2005). An internal positive control synthetic template (ABI, Grand Island, NY) was included in each reaction as an indicator for the presence of PCR inhibitors. Samples for which the internal control for the PCR reaction failed were set as missing.

Analysis

Prevalence of infection with *T. gondii* and CMV were examined overall and by state of birth. Differences in population characteristics between case-infants and control-infants were analyzed using chi-square tests of association or Fisher's exact tests. Data were sparse, but when possible, crude odds ratios (cORs) and confidence intervals (CIs) were calculated using Fisher's exact test to estimate the association between each infection and congenital hydrocephalus. All analyses were performed in SAS 9.3 (Cary, NC).

RESULTS

Demographic Characteristics

In total, 858 DBS were sent to CDC with 410 samples from infants with hydrocephalus (case-infants) and 448 infants with no major birth defects (control-infants). Approximately two-thirds of mothers were <30 years of age, and over three-quarters were non-Hispanic white or Hispanic. Overall, neither maternal age nor maternal race/ethnicity differed by case status (Table 1).

By state of maternal residence, 515 DBS were from infants in CA, 160 were from NC, and 183 were from TX. Case- and control-mothers were not statistically different by maternal age in any of the three sites. There was no significant difference in maternal race/ethnicity

between cases and controls in CA or NC, but in TX, case-infants were more likely to be non-Hispanic Black than control-infants (Table 1).

***T. gondii* Antibody Detection from Residual DBS**

T. gondii IgM testing correctly identified 10 of 10 negative controls (specificity = 100%) and 13 of 13 positive controls (sensitivity = 100%). Of the 245 case-infants from CA, *T. gondii* IgM was detected in three residual DBS (1.2%; Table 2). None of the residual DBS from control-infants had detectable levels of *T. gondii* IgM (Table 2). A frequency of three cases with *T. gondii* infection versus zero controls was not different statistically ($p = 0.11$).

CMV Detection from Residual DBS

CMV DNA testing correctly identified 10 of 10 negative controls (specificity = 100%) and 12 of 13 positive controls (sensitivity = 92%). Samples from all sites were tested for CMV DNA. Seventeen samples were set to missing and excluded from the analysis because the internal control for the PCR reaction failed. After testing for *T. gondii*, three residual DBS from NC had insufficient samples remaining for CMV PCR analysis, yielding a total sample size of 838 DBS analyzed. Overall, six of 396 case-infants with hydrocephalus (1.5%) and three of 442 control-infants without birth defects (0.7%) had residual DBS that tested positive for CMV DNA (cOR: 2.3; $p = 0.32$; 95% CI: 0.48, 13.99; Table 2).

DISCUSSION

Newborns with congenital hydrocephalus were more likely to have evidence of a CMV or *T. gondii* infection at delivery compared with newborns without congenital hydrocephalus, but differences were not statistically significant. The lack of statistical significance might be related to the small number of samples available, the quality of the samples available, or might suggest that a relatively small proportion of congenital hydrocephalus is related to these two infections. A preliminary power analysis for samples of 500 cases and 500 controls, assuming an estimated prevalence among controls of 0.01 (CMV) and 0.002 (*T. gondii*), indicated that the minimum odds ratios detectable for the association of congenital hydrocephalus with either maternal CMV or *T. gondii* were 3.78 and 10.6, respectively. While this pilot study did not identify any statistically significant association of CMV or *T. gondii* with congenital hydrocephalus, it did demonstrate some of the challenges in assessing the role of prenatal infections in the etiology of congenital disorders, which include sample size, quality of samples, and the variation in prevalence of infection between populations. This approach might be most useful when higher quality samples are available and when conducting research in populations with a higher prevalence of these infections.

Birth prevalence of congenital CMV infection in the United States ranges from 0.5 to 1.3%, and CMV is estimated to infect 30,000–40,000 infants annually (Ross and Boppana, 2005; Dollard et al., 2007). Some of the health problems in the neonate associated with intrauterine infection with CMV include chorioretinitis, intrauterine growth restriction, and sensorineural hearing loss and vision impairment (Ornoy and Diav-Citrin, 2006). The rate of newborn infection with *T. gondii* is estimated to be one per 10,000 live births (Jara et al., 2001; Bale, 2002). The adverse effects of intrauterine infection with *T. gondii* on the neonate include

chorioretinitis and cerebral calcifications (Bale, 2002; Ornoy and Diav-Citrin, 2006; Olariu et al., 2011). The prevalence of CMV and *T. gondii* infections vary by demographic factors, with older age and lower socioeconomic status being associated with infection (Jones et al., 2007; Bate et al., 2010). In women with normal immune systems, risk of fetal infection is highest among mothers who acquire a primary CMV or *T. gondii* infection during pregnancy (Bale, 2002; Kravetz and Federman, 2005; Kenneson and Cannon, 2007).

Newborn screening programs in most states store residual DBS for at least one year; at least 14 programs store residual DBS up to 21 years (Therrell and Hannon, 2012). These residual DBS can potentially be used to diagnose retrospectively congenital infections after the occurrence of adverse health outcomes, to screen for congenital infections, or for retrospective population-based analyses of birth defects or other conditions (Choi et al., 2009; Therrell et al., 2011). A challenge to using residual DBS is that storage conditions are often not ideal, thus limiting the research studies that can be performed (Therrell et al., 1996). In addition, the availability of residual DBS for research varies by state (Therrell et al., 2011; Therrell and Hannon, 2012).

This study had several strengths. Case-infants were identified using established population-based birth defects surveillance systems, and control-infants were drawn from the same source population and time period as case-infants. Additionally, abstracted medical records of cases were reviewed by a clinical geneticist to ensure a consistent case-definition for congenital hydrocephalus was used for all case-infants. Appropriate case classification of congenital hydrocephalus is important, as the disease is multifactorial and can arise from numerous causes.

Limitations of the study include lack of ideal storage conditions for ~40% of the residual DBS. *T. gondii* IgM recovery was significantly impaired in samples stored in ambient conditions (Mei et al., 2011), leading to the exclusion of samples from NC and TX for *T. gondii* antibody testing. Moreover, CMV DNA viral load is reduced, although still recoverable, after 2 years (Atkinson et al., 2009). Second, previous studies have indicated that neonatal screening for *T. gondii* IgM using DBS detects ~52–87% of infected newborns (Lebech et al., 1999; Paul et al., 2000; Gilbert et al., 2007). Similarly, the sensitivity of CMV DBS DNA testing approaches 100% when testing symptomatic newborns (Barbi et al., 1996; Snijdewind et al., 2012), but ranges from ~30 to 80% when testing unselected population-based newborns (Soetens et al., 2008; Boppana et al., 2010). While this suggests some case- and control-infants with *T. gondii* or CMV infection might have been missed with an approach that used residual DBS, the study was most likely to miss those with CMV infection who had a low viral load and thus were at low risk for clinical sequelae related to the infection. Third, while this was a population-based study, it was a pilot with a small sample size, and the estimates for the prevalence of *T. gondii* and CMV infections are imprecise. The data were too sparse to be stratified by race/ethnicity, which is problematic given that case-infants from Texas were more likely than controls to be non-Hispanic black and CMV is more common among non-Hispanic blacks (Bate et al., 2010). A larger number of DBS specimens from both case-infants and control-infants, higher quality samples, or a population with a higher prevalence of infection would be needed to better understand the association between congenital hydrocephalus and these infections during pregnancy.

Finally, DBS provide no information regarding maternal infection status before pregnancy or of the timing of the infection during pregnancy. These factors could greatly influence the occurrence and severity of any subsequent sequelae, such as congenital hydrocephalus.

In summary, this pilot study observed results consistent with the hypothesis that *T. gondii* and CMV infections during pregnancy may be more frequent among infants with congenital hydrocephalus compared to unaffected infants, although these results were not significant. Future studies using DBS from larger samples of case-infants and control-infants would be needed to understand better the potential contribution of *T. gondii* or CMV infection during pregnancy to the occurrence of congenital hydrocephalus in the population. Residual DBS represent an important, population-based resource that can be used to understand better the association between infections during pregnancy and adverse birth outcomes including major birth defects. For infections that are predominantly asymptomatic or present with nonspecific symptoms, objective testing such as that available using DBS will be one of the few paths available to evaluate the etiologic role of these infections. Such studies would be facilitated by more widespread adoption by state newborn metabolic screening programs of the recommended procedures for storing residual DBS.

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Table 1
Demographic Characteristics of Infants with Hydrocephalus and Infants without Major Birth Defects with DBS Specimens from California, North Carolina, and Texas

	Overall (N = 858)		California (N = 515) ¹		North Carolina (N = 160) ²		Texas (N = 183) ³	
	n	(%)	n	(%)	n	(%)	n	(%)
Maternal Age								
<30	269	(65.6)	155	(63.3)	179	(66.3)	55	(73.3)
30	141	(34.4)	90	(36.7)	91	(33.7)	20	(26.7)
		<i>p</i> = 0.89		<i>p</i> = 0.47		<i>p</i> = 0.08		<i>p</i> = 0.87
Maternal Race/Ethnicity								
White, Non-Hispanic	144	(35.1)	168	(37.5)	66	(26.9)	75	(27.8)
Black, Non-Hispanic	58	(14.1)	41	(9.2)	23	(9.4)	18	(6.7)
Hispanic	172	(42.0)	201	(44.9)	128	(52.2)	148	(54.8)
Other	36	(8.8)	38	(8.5)	28	(11.4)	29	(10.7)
		<i>p</i> = 0.14		<i>p</i> = 0.70		<i>p</i> = 0.89		<i>p</i> = 0.007

¹ California dried blood spots were stored in temperature- and humidity-controlled (-20 °C, < 30% humidity) conditions with desiccant. Blood spots were collected between 1995 and 2003.

² North Carolina dried blood spots were stored under ambient conditions (1-40 °C) without desiccant. Blood spots were collected between 2003 and 2005.

³ Texas dried blood spots were stored under ambient conditions (1-40 °C, in a dehumidified laboratory) without desiccant. Blood spots were collected between 2003 and 2004.

Table 2
T. gondii IgM and CMV DNA Testing Results from DBS of Newborns with Congenital Hydrocephalus (Cases) Matched to Newborns without Major Birth Defects (Controls)

	Overall (N = 855)		California (N = 515)		North Carolina (N = 157) ¹		Texas (N = 183) ¹	
	Case n (%)	Control n (%)	Case n (%)	Control n (%)	Case n (%)	Control n (%)	Case n (%)	Control n (%)
<i>T. gondii</i>								
Positive	3 (1.2)	0 (0.0)	3 (1.2)	0 (0.0)	2 (3.0)	0 (0.0)	1 (1.1)	2 (2.2)
Negative	242 (98.8)	270 (100.0)	239 (98.8)	267 (99.6)	64 (97.0)	81 (100.0)	87 (98.9)	91 (97.9)
CMV								
Positive	6 (1.5)	3 (0.7)	3 (1.2)	1 (0.4)	2 (3.0)	0 (0.0)	1 (1.1)	2 (2.2)
Negative	390 (98.5)	439 (99.3)	239 (98.8)	267 (99.6)	64 (97.0)	81 (100.0)	87 (98.9)	91 (97.9)
Missing	12	5	3	2	7	3	2	0

¹ Samples from North Carolina and Texas were excluded from *T. gondii* IgM results due to poor IgM recovery, likely because of sample degradation in ambient storage conditions.