THE IMPACT OF GB VIRUS C CO-INFECTION ON MOTHER TO CHILD TRANSMISSION OF HUMAN IMMUNODEFICIENCY VIRUS

by

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ABSTRACT

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GB virus C (GBV-C) is a common, apathogenic virus that can inhibit human immunodeficiency virus (HIV) replication in vitro. Persistent coinfection with GBV-C has been associated with improved survival among HIV-infected adults while loss of GBV-C viremia has been associated with poor survival. If GBV-C does inhibit HIV replication, it is possible that GBV-C infection may reduce mother-to-child-transmission (MTCT) of HIV. This study investigated whether maternal or infant GBV-C infection was associated with reduced MTCT of HIV infection.

The study population consisted of 1,783 pregnant women from three Bangkok perinatal HIV transmission studies (1992-94, 1996-7, 1999-2004). We tested plasma collected at delivery for GBV-C RNA, GBV-C antibody, and GBV-C viral genotype. If maternal GBV-C RNA was detected, the four- or six-month infant specimen was tested
for GBV-C RNA. Rates of MTCT of HIV in GBV-C-infected and GBV-C-uninfected women and infants were compared using multiple logistic regression as were associations with MTCT of GBV-C and prevalence of GBV-C infection.

The prevalence of GBV-C infection (i.e. presence of RNA or antibody) was 33% among HIV-infected women and 15% among HIV-uninfected women. Forty-one percent of GBV-C-RNA-positive women transmitted GBV-C to their infants. Only two of 101 (2.0%) GBV-C-RNA-positive infants acquired HIV infection compared to 162 (13.2%) of 1,232 of GBV-C-RNA-negative infants (RR 0.15, p<0.0001). This association remained after adjustment for maternal HIV viral load, antiretroviral prophylaxis, CD4+ count and other covariates. MTCT of HIV was not associated with presence of maternal GBV-C RNA or maternal GBV-C antibody. Maternal receipt of antiretroviral therapy was associated with increased MTCT of GBV-C, as was high GBV-C viral load, vaginal delivery and absence of infant HIV infection. GBV-C infection among women was independently associated with more than one lifetime sexual partner, intravenous drug use and HIV-infection.

We observed a higher prevalence of GBV-C infection among HIV-infected compared to HIV-uninfected pregnant women in Thailand, likely due to common risk factors. Antiretroviral therapy appears to increase MTCT of GBV-C. Infant GBV-C acquisition, but not maternal GBV-C infection, was significantly associated with reduced MTCT of HIV. Mechanisms for these later two associations are unknown.
Dedication

I would like to dedicate this thesis to my family: to my husband who felt that I deserved the opportunity to complete the PhD program; to my parents, especially my mother, whose unending support at home allowed me to complete the program no matter what else was happening; and, to my five children, Chris, Nick, Emma, Allison, and Jennifer, who could never understand why their mother was still in school, but who provided numerous joys and crises along the way that alternately sustained me and kept me grounded in real life.
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Glossary of Terms

**AIDS** - acquired immune deficiency syndrome, the advanced form of infection with the HIV virus. This includes a drop in CD4+ cell count and/or one of several illnesses considered to be ‘AIDS defining’

**ART** - antiretroviral therapy (ART), ARVs used to treat or prevent HIV infection

**ARV** - antiretroviral (ARV), several classes of drugs that fight retroviruses. These include the NRTIs (nucleoside reverse transcriptase inhibitors), NNRTIs (non-nucleoside reverse transcriptase inhibitors), PIs (protease inhibitors), and integrase inhibitors. Other newer agents act to prevent entry of the virus into the cell including fusion inhibitors and CCR5 antagonists.

**AZT** = ZDV = Zidovudine, an NRTI, was the first drug licensed to treat HIV. It was also the first antiretroviral used to prevent mother-to-child-transmission (MTCT) of HIV. It is still widely used globally for the prevention of MTCT of HIV.

**CD4+ cell** - CD4+ cells are one of the T helper lymphocytes and are important for immune activation. HIV replicates in CD4 cells and enters a CD4+ cell via a CD4 receptor and a coreceptor (most commonly CCR5, or CXCR4).

**CD4+ count** – Normal CD4+ counts are between 500 and 1600 and are a measure of immune function. CD4+ cells become depleted during HIV progression and CD4+ counts are used for HIV staging (<200 cell/mm³ = stage 3 = AIDS, 200-499 = stage 2 and, >500 = stage 1, along with other disease diagnoses (CDC criteria).

**HAART** - highly active antiretroviral therapy (HAART) (triple therapy), an approach typically involving three ARVs taken in combination. This is the standard of care for treatment of HIV and for the prevention of mother-to-child-transmission in Western developed countries.

**HIV** – human immunodeficiency virus (HIV) is a retrovirus that causes AIDS by infecting T cells of the immune system. The most common serotype, HIV-1, is distributed worldwide, while HIV-2 is primarily confined to West Africa.

**HIV viral load** – HIV viral load tests measure the number of HIV viral particles per mm³ of blood and is a measure of disease severity. HIV viral load results are useful for diagnosis, managing therapy (deciding when to start taking or to change antiretrovirals), prevention (those with high viral loads are most likely to transmit virus), and prognosis (those with high viral loads are likely to progress to AIDS more rapidly).
**Retrovirus** – an RNA virus that replicates by first being converted into double-stranded DNA by reverse transcriptase.

Chapter 1
Background & Objectives

1.1 INTRODUCTION

More than 2 million women infected with human immunodeficiency virus (HIV) become pregnant worldwide each year [McIntyre, 2005] and, despite the advent of antiretroviral prophylaxis and other interventions which can greatly reduce the risk of mother-to-child transmission (MTCT) of HIV, approximately 420,000 children became HIV-infected in 2007. Failure to provide maternal antiretroviral therapy (ART) and high maternal HIV viral load are the main risk factors for MTCT of HIV; others include mode of delivery, duration of ruptured membranes, female sex of the infant, and breastfeeding. Improved knowledge regarding biologic mechanisms that inhibit HIV replication may aid in the development of pharmaceutical agents to prevent MTCT of HIV and may also aid the development of other forms of prevention and treatment of HIV in general.

GB virus C (GBV-C), a flavivirus closely related to hepatitis C virus (HCV) [Berzsenyi et al., 2005b] and not known to cause any disease, may have an inhibitory effect on HIV-1 replication. Coinfection with GBV-C has been reported to have a beneficial effect on HIV progression and survival in some but not all studies [Birk et al.,
Several studies have also observed an association with decreased HIV viral load and/or increased CD4+ count [Custer et al., 2004; Heringlake et al., 1998; Lefrere et al., 1999c; Li et al., 2006a; Nunnari et al., 2003; Tillmann et al., 2001; Yeo et al., 2000; Yirrell et al., 2007]. Three of four recent longitudinal studies observed that persistent coinfection with GBV-C virus was associated with improved survival in HIV-infected adults, while loss of GBV-C RNA was associated with worse survival in all studies [Bjorkman et al., 2004; Van der Bij et al., 2005; Williams et al., 2004; Yirrell et al., 2007]. A meta-analysis of six studies supports a beneficial effect of GBV-C on HIV-1 progression after coinfection lasting at least two years [Zhang et al., 2006]. Some investigators [Stapleton et al., 2004; Williams et al., 2004] have suggested that GBV-C infection may slow HIV progression by inhibiting HIV replication while others [Bjorkman et al., 2004; Van der Bij et al., 2005] believe that causation is likely reversed and that GBV-C infection status is, rather, secondary to HIV progression, possibly mediated through CD4+ cell depletion. However, in vitro studies have demonstrated that infection of cell cultures with GBV-C inhibits HIV replication [Jung et al., 2005; Xiang et al., 2004; Xiang et al., 2001] and GBV-C appears to replicate in cells other than CD4+ cells [George et al., 2006] making the CD4+ hypothesis less likely. Still, the nature of the interaction between HIV and GBV-C remains unclear.
If GBV-C does inhibit HIV replication, then it is possible that maternal or infant infection with GBV-C might reduce MTCT of HIV. The present study tests this hypothesis using data and stored blood from three large perinatal studies in Bangkok and also examines the prevalence and correlates of maternal GBV-C infection among pregnant women in Thailand and the rate and correlates of MTCT of GBV-C among GBV-C-HIV coinfected women.

1.2 BACKGROUND AND LITERATURE REVIEW

1.2.1 THE GB VIRUS C (GBV-C)

Hepatitis GB virus C (HGBV-C/GBV-C) and hepatitis G virus (HGV) were discovered almost simultaneously in 1995 and 1996 by two independent research teams [Linnen et al., 1996; Simons et al., 1995] attempting to identify causative agents of non-A-E viral hepatitis. These two viruses were later shown to be almost identical isolates of the same virus (86% at the nucleotide level and 97% at the amino acid level) [Kleinman, 2001]. One team identified a ‘GB agent’ in a surgeon with initials G.B. who had developed hepatitis in the 1960s [Simons et al., 1995]. Transfusion of his plasma to tamarins resulted in clinical hepatitis [Deinhardt et al., 1967]. Subsequent analysis of the tamarin serum revealed two different viral species, GBV-A and GBV-B [Muerhoff et al., 1995] but these were eventually found to be non-human marmoset viruses [Alter, 1996; George et al., 2002]. The human virus, HGBV-C/GBV-C, was subsequently discovered
by gene amplification with primers derived from the shared sequences in GBV-A, GBV-B and HCV in patients with non-A non-B hepatitis [Simons et al., 1995]. HGV was cloned in a similar manner by another research team from a patient with community acquired chronic non-A through E hepatitis whose plasma also transmitted hepatitis to tamarins [Linnen et al., 1996]. Because this virus has not been found to cause liver disease in humans, it is now generally referred to as ‘GB virus C’ (GBV-C).

Phylogenetically, GBV-C is most closely related to the hepatitis C virus, with approximately 30% amino acid homology [Berzsenyi et al., 2005b] (Figure 1). GBV-C and HCV are separate genera within the Flaviviridae family [Alter, 1996]. Like HCV, the GBV-C genome is a positive sense single stranded RNA, approximately 9,400 nucleotides long, coding for the envelope and non-structural proteins; GBV-C does not have a core protein [Kleinman, 2001].
Because GBV-C was initially found in patients with liver disease, it was assumed that GBV-C was responsible for at least some cases of non-A-E viral hepatitis. However, a virus common to the general population would be found in patients with a variety of diseases and extensive investigation has revealed no association between GBV-C and liver disease. Because GBV-C has not yet been associated with any disease in humans [Alter, 1996; Kleinman, 2001], it has been referred to as a human orphan virus [George et al., 2002].

The site of GBV-C replication in the body is not exactly known. Despite GBV-C RNA having been detected in hepatocytes in some studies [George et al., 2002], GBV-C is generally thought to be a panlymphotrophic virus [George et al., 2006]. Comparisons
between GBV-C and HCV RNA concentrations in liver tissue and plasma demonstrate that, while HCV RNA is found predominantly in the liver, GBV-C RNA is found predominantly in plasma, suggesting that HCV replicates in the liver while GBV-C does not [Pessoa et al., 1998]. GBV-C has also been observed to replicate in vitro in peripheral blood mononuclear cells (PBMCs) [Xiang et al., 2001], including CD4+ and CD8+ T lymphocytes, and CD19+ B lymphocytes [George et al., 2006]. However, it does not replicate well [George et al., 2003], leading some to suggest that PBMCs are at most a minor reservoir of GBV-C replication [Mellor et al., 1998]. Results from a cadaveric study suggested that GBV-C is primarily lymphotropic, with replication mainly in the spleen and bone marrow [Tucker et al., 2000].

GBV-C and other flaviviruses such as HCV are thought to enter cells primarily through low density lipoprotein receptors [Agnello et al., 1999]. HIV, on the other hand, uses the CD4 receptor with chemokine coreceptors chemokine (C-C motif) receptor 5 (CCR5) and chemokine (C-X-C motif) receptor 4 (CXCR4) to enter cells [The Body, 2003].

The GBV-C virus is currently known to have six genotypes whose global distribution is thought to correlate with geographic origin at the time the human races diverged suggesting an ancient and slow-mutating virus [Smith et al., 2000]. The six genotypes are thought to be found predominantly in the following regions: genotype 1, West Africa; genotype 2, North America and Europe; genotype 3, Asia; genotype 4, Southeast Asia; genotype 5, South Africa [Tucker and Smuts, 2000]; and genotype 6, the
latest to be discovered, in Indonesia [Muerhoff et al., 2006]; however this is based on limited studies. Within some genotypes, distinct subtypes have also been identified. Although there are six genotypes, GBV-C shows much less genetic variation than HCV [Kleinman, 2001].

1.2.2 TRANSMISSION AND OCCURRENCE OF GBV-C INFECTION

GBV-C is a blood-borne virus that can be transmitted parenterally, sexually, and vertically [Kleinman, 2001]. It is not known if GBV-C can also be transmitted through casual contact. GBV-C RNA has been found in blood, saliva, and semen [Bourlet et al., 2002] but not urine [Seemayer et al., 1998] or breast milk [Schroter et al., 2000]. Rates of GBV-C viremia vary with geographic location and prevalence increases with sexual and parenteral risk behaviours.

Rates of GBV-C infection in pregnant women, a proxy for rates in the general population, tend to be lowest in East Asia (1-2%) [Lin et al., 1998; Ohto et al., 2000], intermediate in Europe, Australia, North America and Southeast Asia (5-7%) [Hyland et al., 1998; Lefrere et al., 1999b; Poovorawan et al., 1998; Skidmore and Collingham, 1999], and highest in Africa (10-13%) [Liu et al., 2000; Tuveri et al., 2000]. Given the geographic variation in both genotype and prevalence of GBV-C, it is possible that certain genotypes are more easily transmissible than others or, alternately, that this apparent association is confounded by risk behaviours.
Prevalence of GBV-C viremia by parenteral and sexual risk group ranges globally from 1 to 4% among blood donors [Kleinman, 2001], 1 to 13% in antenatal populations [Hyland et al., 1998; Lin et al., 1998; Liu et al., 2000; Ohto et al., 2000; Skidmore and Collingham, 1999; Tuveri et al., 2000], 10 to 24% among anti-HCV positive persons [Hino et al., 1998; Katayama et al., 1997; Wejstal et al., 1999; Zanetti et al., 1998], 17 to 24% among hemophiliacs [Toyoda et al., 1998; Yeo et al., 2000], 17 to 44% among HIV-infected persons [Tillmann et al., 2001; Xiang et al., 2001; Zanetti et al., 1998], and 20 to 49% among intravenous drug users (IDUs) [Hyland et al., 1998; Katayama et al., 1997] [de Martino et al., 1998; Saganuma et al., 1998; Vimolket et al., 1998]. Some groups of IDU and MSM have evidence of nearly universal infection [Stapleton, 2003].

GBV-C is transmitted sexually and sexual transmission appears to be a common transmission route for GBV-C. A study from Taiwan reported that 42% of spouses of GBV-C infected persons were also GBV-C infected [Kao et al., 1997]. Other studies have observed an association between GBV-C infection and increased number of sexual partners [Bjorkman et al., 2001; Ribeiro-dos-Santos et al., 2002; Wu et al., 1997] and a history of sexually transmitted diseases [Bjorkman et al., 2001; Tan et al., 1999]. Several studies have reported very high rates of GBV-C infection among homosexual men and/or associations between GBV-C infection and male homosexuality [Berzsenyi et al., 2005a; Lefrere et al., 1999b; Rey et al., 2000; Scallan et al., 1998; Stark et al., 1999]. One study estimated an annual incidence rate of 11% among Scottish gay men [Scallan et al., 1998]. Another study reported an association between persistence of GBV-C infection and male-
male sex in those with advanced HIV disease [Sheng et al., 2007]. Male homosexual transmission may be more efficient than heterosexual transmission based on one study [Berzsenyi et al., 2005a]. Among heterosexuals, transmission from male to female may be more efficient than female to male based on another [Lefrere et al., 1999b].

Several studies have identified parenteral routes of infection, including injecting drug use (IDU) [Christensen et al., 2003; Katayama et al., 1997; Lefrere et al., 1999b; Saganuma et al., 1998; Tan et al., 1999] and receipt of blood transfusions [Henrichsen et al., 2002; Stark et al., 1997; Tan et al., 1999; Vanhems et al., 2003], as risk factors for GBV-C infection. The efficiency of parenteral transmission of GBV-C appeared to be high in one study, with 58% of recipients of GBV-C infected blood products becoming infected [Seifried et al., 2004]; however, other studies have observed a low efficiency of GBV-C transmission by blood components [Lefrere et al., 1999b]. Lefrere et al. observed twice the prevalence of GBV-C RNA in those at high risk for sexually transmitted viruses compared to those at high risk for blood-borne viruses [Lefrere et al., 1999b]. While parenteral routes (IDU and blood transfusion) are more uniquely associated with HCV transmission, for GBV-C, sexual routes were equally efficient in several studies [Bourlet et al., 1999; Ibanez et al., 1998; Nerurkar et al., 1998; Ramia et al., 2004].

Other reported risk factors include health care work [Christensen et al., 2003], hospitalization for various medical procedures such as delivery and abortion [Lefrere et al., 1999b], endoscopy [Bjorkman et al., 2001], and bronchoscopy [Vanhems et al.,]
2003], as well as a history of schistomiasis [Hassoba et al., 1997], exposure to blood-sucking insects [Ribeiro-dos-Santos et al., 2002], snorting cocaine, and imprisonment [Tan et al., 1999].

GBV-C viremia is common among HIV-infected populations likely due to similar routes of transmission [Stapleton et al., 2004]. HIV-infection status did not appear to influence GBV-C acquisition on one study [Williams et al., 2005] and GBV-C infection status did not appear to influence HIV acquisition in another [Bisson et al., 2005]. Interestingly, in the later study of HIV seroconverters, GBV-C prevalence increased from 16% before HIV acquisition to 38% after HIV infection, raising the possibility that the two viruses may be acquired simultaneously [Bisson et al., 2005].

Several studies have reported that the distribution of GBV-C viremia varies with age. Prevalence in the general population appears to increase dramatically at the time sexual activity begins and levels off in middle adulthood. Among the black general population in South Africa, the distribution of viremia by age was reported to be 9% among those aged 15 years or less, 29% among those aged 16-35, and 25% among those 36 or older in one study [Mphahlele et al., 1999]. In Denmark, prevalence was only 1.4% in children with no significant difference in prevalence between 9 and 15 year olds. This increased to 2.2-3.6% in the 18-29 year age group, 3.2-3.6% among 30-39 years olds, and began declining after age 40 [Christensen et al., 2003]. An Australian study of well children with no parenteral risk of exposure reported a prevalence rate of only 1.3% among children aged 2 months to 15 years [Siebert et al., 2002] which is considerably
lower than the 5.0% observed in an earlier Australian study among children admitted through emergency departments (who may have illnesses compatible with frequent hospitalizations and transfusions) [Hyland et al., 1998]. Rates of GBV-C viremia also appeared to increase with age in one study of children with no history transfusion admitted to a pediatric hospital in Washington D.C. where 0/41 (0%) infants less than 1 year old, 4/44 (9%) children aged 1-5 years, 1/32 (3%) aged 6-10 years, 3/33 (9%) children aged 11-15 years and 2/20 (10%) aged 16-20 years had evidence of virus; a significant increase was observed after age 10 [Handa et al., 2000]. In West African children diagnosed with anemia related to malaria, prevalence of GBV-C RNA and antibody increased steadily from 2.0% at 6 months to 13% at 3 years of age (blood samples taken prior to transfusion) [Li et al., 2006b] but it is not known if the increased prevalence was related to previous transfusions or other parenteral risk factors. Among IDUs, the pattern may be slightly different from the general population as both a Thai and Danish study found that GBV-C RNA was most prevalent among younger IDUs (18-30 year olds) and decreased with age [Christensen et al., 2003; Vimolket et al., 1998].

Although studies to date have been small and have given a wide range of estimates, MTCT of GBV-C appears to be common and several studies have observed rates as high as 50-80% [Hino et al., 1998; Lefrere et al., 2000; Lin et al., 1998; Ohto et al., 2000; Wejstal et al., 1999; Zanetti et al., 1998]. This compares with MTCT rates of 3-7% for HCV its closest relative [Herrine, 2006; Wejstal et al., 1999] and 14-42% for HIV [Kourtis et al., 2001]. HTLV-1, a retrovirus, is transmitted primarily through breastfeeding and not during delivery; intrauterine transmission is rare [Fujino and Nagata,
The prevalence of GBV-C is higher in the general population and blood donors (in western industrialized countries) than other blood-borne viruses such as hepatitis C and HIV, and high rates of intrafamilial, vertical, and sexual transmission have been suggested as a possible explanation [Fischler et al., 1997; Hino et al., 1998; Pinho et al., 1999]. Vertical transmission of GBV-C is discussed further below.

Among HIV-infected children, GBV-C infection appears to be less common than in HIV-infected adults. In one U.S. study, GBV-C RNA prevalence among HIV-infected children was 2.1% in those less than 6 years of age and increased with age to 4.4% in those 6-13 years of age, to 9.7% in those 13 or older [Schuval et al., 2005], possibly explained by increased exposure to blood components as HIV-infected children are frequently diagnosed with anemia [Kourtis et al., 2006]. Other studies have also observed low rates of GBV-C-HIV coinfection in children. Only 3/60 (5%) of Kenyan HIV-infected children aged 1-18 [Chakraborty et al., 2003], 0/37 (0%) of Italian HIV-infected children aged 8 months to 16 years [Pontali et al., 2000], and 20/353 (6%) of American HIV-infected children aged one to 21 years [Schuval et al., 2005] were infected with GBV-C.

The prevalence of the antibody to the GBV-C envelope protein E2 (anti-E2) is usually about twice that of viremia in immunocompetent persons [Lefrere et al., 1999b], with rates of anti-E2 varying in low-risk populations from approximately 10% in blood donors [Heringlake et al., 1998; Hyland et al., 1998] in Australia and Europe to 5-40% in antenatal populations globally [Hyland et al., 1998; Skidmore and Collingham, 1999;
Tuveri et al., 2000]. In high-risk populations, anti-E2 prevalence ranges from 39%-57% in anti-HCV positive cohorts [Hyland et al., 1998; Wejstal et al., 1999] and 15-57% in HIV-positive cohorts [de Martino et al., 1998; Lefrere et al., 1999c; Tillmann et al., 2001]. Several studies have observed that prevalence of antibody to GBV-C increases with age [Feucht et al., 1999; Rey et al., 2000], likely a function of duration of infection. One study observed a higher seroprevalence of GBV-C antibody from 6% in childhood, to 15% in mid teens, and to 25% around the age of 40 [Feucht et al., 1999].

### 1.2.3 NATURAL HISTORY OF GBV-C VIRUS: PERSISTENCE AND CLEARANCE

GBV-C is generally detectable in adults two to three weeks after infection [Alter et al., 1997; Sauleda et al., 1999] with a high persistent viral RNA level observed (10^5-10^7 viral copies/ml) [Lefrere et al., 1999a; Ohto et al., 2000] and no apparent decline prior to viral clearance [Lefrere et al., 1999a]. GBV-C viremia often persists for years but is cleared eventually in 50-75% of people, at which time antibodies to the GBV-C E2 envelope usually appear [Kleinman, 2001]. In contrast, fewer than 25% of HCV-infected persons spontaneously clear infection [Stapleton et al., 2004]. Infections, however, can last for many years, making it difficult to get a true picture of the natural history of GBV-C, as most studies have not followed subjects long enough to determine duration of infection. One study with an average follow-up-time of 5.6 years showed a mean infection time of 4-5 years, although several were positive for 9 years, and most observations were censored (either no start date or no stop date) [Lefrere et al., 1997].
Several longitudinal studies of blood transfusion recipients and hemophilic patients demonstrated clearance of the virus in 50% of persons infected for one to six years [Kleinman, 2001]. One study reported a rate of RNA loss of 18% and E2 antibody loss of 15% over 3 years but time of infection was unknown [Busch et al., 2003].

It has been suggested that younger persons and those who are immunocompromised or have coinfections may be less able to clear the virus [Hardikar et al., 1999; Lin et al., 1998; Thomas et al., 1998; Zuin et al., 1999]. Devereux et al. observed that HIV-uninfected haemophilic patients were more likely to clear GBV-C RNA than HIV-infected patients [Devereux et al., 1998]. A lower ratio of anti-E2:GBV-C RNA (1:4) in children compared to adults (2:1) is suggestive of slower clearance in younger children [Hardikar et al., 1999]. Clearance of GBV-C in infants rarely occurs under one year of age but a high proportion generally become RNA-negative and anti-E2 positive by 3.5 years of age [Zuin et al., 1999]. In one study with a long follow-up, 29% of neonatally transfused infants who became GBV-C-infected remained viremic 15 years later, indicating a clearance rate in children of 70% over 14 years [Berg et al., 1997]. Results of another study examining viral persistence and antibody responses in children suggested that infants acquiring GBV-C perinatally may be less able to mount an immune response than those acquiring GBV-C from blood and that mode of transmission and age at infection may be important factors in determining persistence of GBV-C and immune response [Chen et al., 1998]. Of 17 children infected with GBV-C at birth, only one infant converted to RNA-negative status at 24 months. In comparison, of 14 children
aged six months to 14 years infected with GBV-C via blood transfusion, eight cleared GBV-C infection and four of these eight developed antibodies [Chen et al., 1998].

In a cohort of injection drug users, many of whom also had markers of HBV, HCV, and HIV infection, GBV-C RNA-positive adults experienced a clearance rate of only 20% (8/40) in 6.5 years of follow-up [Thomas et al., 1998]. However, a study of HIV-infected women receiving highly active antiretroviral therapy (HAART) reported that viral clearance was somewhat higher at 6.3 per 100 person-years after a median follow-up of 6.5 years. The authors felt that HAART may have strengthened these women’s immune systems, permitting more rapid viral clearance at a rate similar to that of HIV-uninfected women [Williams et al., 2005]. HCV interferon therapy also enhances GBV-C RNA clearance [Schwarze-Zander et al., 2006].

When the virus is cleared, antibody to the GBV-C envelope protein E2 (anti-E2) usually, but not always, appears [Kleinman, 2001]. Presence of GBV-C antibody is therefore generally a serologic marker for recovery [Tacke et al., 1997] similar to hepatitis B and dissimilar to hepatitis C [Kleinman, 2001]. GBV-C RNA and anti-E2 are found simultaneously in <5% of GBV-C viremic persons [Hyland et al., 1998; Lefrere et al., 1999b; Tacke et al., 1997; Wejstal et al., 1999; Yeo et al., 2000; Zanetti et al., 1998]; however, when they do occur together, the virus has been shown to subsequently clear [Lefrere et al., 1997; Thomas et al., 1998]. Two studies have observed that antibody to GBV-C protects against reinfection [Busch et al., 2003; Thomas et al., 1998] and, even if antibodies are lost, protection against reinfection apparently remains [Busch et al., 2003].
There have also been several reports of loss of GBV-C viremia without detection of GBV-C antibodies among HIV-coinfected adults [Bjorkman et al., 2004; Devereux et al., 1998; Stapleton et al., 2004; Van der Bij et al., 2005], although it is possible that antibodies may have been evident after clearance but lost over the study period. Two studies have observed that loss of GBV-C antibody was higher among HIV-infected than HIV-uninfected men [Devereux et al., 1998; Stark et al., 1999]. In three longitudinal studies of GBV-C HIV-coinfection, most participants who cleared GBV-C did not have detectable antibody at a median of four to six years later including 11/14 from the Bjorkman study [Bjorkman et al., 2004], 9/12 from the MACS cohort [Stapleton et al., 2004], and 64/78 from the Van der Bij study [Van der Bij et al., 2005]. Most of these participants subsequently died.

1.2.4 MECHANISM OF VERTICAL TRANSMISSION OF BLOOD-BORNE VIRUSES

Possible mechanisms for vertical transmission of blood-borne infections in utero or during delivery include maternal-fetal transfusions that occur with uterine contractions, early rupture of membranes allowing direct fetal contact with maternal secretions and blood, and exposure of the infant’s skin and mucosa to maternal blood and cervicovaginal secretions during delivery [Kourtis et al., 2001]. In addition, transmission of some viruses such as HIV can occur after delivery through breast feeding. Maternal viral load is the most important determinant in the vertical transmission of HCV, HBV, HTLV-1, HIV, and probably GBV-C [Ohto et al., 2000]. In addition to viral
virulence and maternal plasma viral load, the efficiency of transmission of blood-borne viruses depends upon the amount of maternal virus that the infant is exposed to transplacentally or through cervicovaginal secretions. Transplacental leakage is thought to be the main cause of in utero infection of HBV and the same is probably true of HCV, GBV-C, and HIV [Lin et al., 1996b]. While the placenta and fetal membranes normally act as a protective barrier for the fetus, disruption of this barrier may occur as the placenta separates with uterine contractions, causing some mother-to-fetus microtransfusion [Lin et al., 1996b]. Elective caesarean section has been shown to cause the least amount of microtransfusion compared to other modes of delivery [Lin et al., 1996a]. Elective caesarean section can therefore prevent HIV transmission late in utero as well as during the delivery [Kourtis et al., 2001]. Vaginal delivery and duration of ruptured membranes greater than four hours prior to delivery all increase exposure to cervicovaginal secretions and have been associated with increased transmission of HIV [Bulterys, 2000].

1.2.5 VERTICAL TRANSMISSION OF GBV-C

Wide variations in rates of MTCT of GBV-C (from 13-100%) have been reported in 19 published studies identified [Barqasho et al., 2004; de Martino et al., 1998; Feucht et al., 1996; Fischler et al., 1997; Hino et al., 1998; Lefrere et al., 2000; Li et al., 2006b; Lin et al., 1998; Menendez et al., 1999; Moaven et al., 1996; Ohto et al., 2000; Palomba et al., 1999; Sathar et al., 2004; Schroter et al., 2000; Tanaka et al., 1997; Viazov et al.,
A limitation of all of these studies is their sample size, varying from one to 34 GBV-C-infected women.

High maternal GBV-C viral load and mode of delivery were identified as risk factors for vertical transmission of GBV-C in three and two of the studies respectively [Hino et al., 1998; Lin et al., 1998; Ohto et al., 2000]. Ohto et al. reported that elective caesarean section compared to vaginal or emergency caesarean section was highly protective against GBV-C vertical transmission on a one tailed t-test (OR 0.13; 95% CI, 0.02-0.82) [Ohto et al., 2000]. High maternal GBV-C viral load was also associated with vertical transmission (mean $10^{6.3}$ viral copies/ml in transmitting mothers compared to $10^{4.6}$ viral copies/ml in non-transmitting mothers; p<0.001) and 96% (23/24) of mothers with a viral load $\geq10^6$ viral copies/ml transmitted GBV-C to their infants [Ohto et al., 2000]. Hino et al. also observed significantly higher GBV-C maternal viral titres in mothers of GBV-C-infected children compared to mothers of GBV-C-uninfected children ($10^{6.7}$ vs. $10^4$ viral copies/ml, p=0.001) [Hino et al., 1998]. Lin et al. found that 13 of 13 mothers of GBV-C-infected children had plasma GBV-C viral loads of greater than $10^7$ viral copies/ml and that mode of delivery was either vaginal or emergency caesarean section [Lin et al., 1998]. In comparison, 10 of 12 mothers of GBV-C-uninfected infants had viral titres $<10^5$ viral copies/ml and the two infants whose mothers had GBV-C viral copies $>10^7$/ml were born by elective caesarean sections. The fact that none of the babies born by elective caesarean section became infected provides further evidence of infection occurring at the time of delivery.
The relative timing of GBV-C and HIV perinatal transmission is important for the present study. GBV-C RNA becomes detectable between birth and 3 months of age. Early detection of viruses such as HIV and HCV in infants (i.e. within the first 48-72 hours) has been used as an indication of in utero viral transmission [Bryson et al., 1992; Kourtis et al., 2001; Schroter et al., 2000], although it is not known if this indication truly distinguishes between infections acquired at different times [Dunn et al., 2000]. GBV-C RNA was detected at birth in some (5-100%) of infants in almost all studies where infants were tested at birth [de Martino et al., 1998; Lefrere et al., 2000; Moaven et al., 1996; Schroter et al., 2000; Viazov et al., 1997; Wejstal et al., 1999; Zanetti et al., 1998]. The only exceptions include one study with nine infected infants where 0/5 tested positive at birth [Palomba et al., 1999] and three studies with only one or two GBV-C infected children [Fischler et al., 1997; Moaven et al., 1996; Sathar et al., 2004]. The wide range in rates of early detection may reflect the small study sample sizes or the lack of a standardized sensitive PCR test for low levels of GBV-C virus. GBV-C RNA was detected in almost all GBV-C-infected infants by two to three months of age in studies where infants were tested at that age [Fischler et al., 1997; Palomba et al., 1999; Viazov et al., 1997; Wejstal et al., 1999; Zanetti et al., 1998; Zuin et al., 1999]. The time when GBV-C is detectable is similar to that of HIV, which can be detected by PCR in 25-40% of infected infants at birth [Biggar et al., 1997; Mock et al., 1999; Young et al., 2000] (this proportion may be increasing with increased prevention of transmission at delivery) and in the remainder by one to two months of age [Young et al., 2000]. As the PCR test for GBV-C requires a higher viral load for reliable detection (1,000 copies per reaction or 50,000 copies/mL) than HIV (50 copies/mL), it may take slightly longer for GBV-C viral
replication to reach a detectable level in the infant. As HIV is thought to be
transmitted primarily late in pregnancy or during delivery in non-breastfeeding women
[Kourtis et al., 2001], a similar timing is likely for GBV-C transmission.

Viral sequence homology between mothers and infants is also an indicator of
vertical transmission. Most but not all GBV-C vertical transmission studies observed
high sequence homology between all mother and infant pairs [Fischler et al., 1997; Hino
et al., 1998; Li et al., 2006b; Lin et al., 1998; Viazov et al., 1997; Wejstal et al., 1999]. In
addition, most also found no GBV-C RNA in the plasma of infants of RNA-negative
mothers [Feucht et al., 1999; Lefrere et al., 2000; Wejstal et al., 1999] implying that it is
unlikely for infants to acquire GBV-C from someone besides the mother. GBV-C
familial studies have also demonstrated virtually 100% sequence homology between
mothers and children [Cheng et al., 2003; Mizutani et al., 2000; Pinho et al., 1999;
Seifried et al., 2004]. Sequence homology was found to be significantly higher within
families (98.6% to 100%) than between families (87.8% to 92.4%) [Cheng et al., 2003],
although the timing of such interfamilial transmission cannot be determined.

In spite of the above, one study from Africa observed low GBV-C sequence
homology in some mother-infant pairs [Menendez et al., 1999] and two noted the
presence of GBV-C RNA in infants of GBV-C RNA-negative mothers [Menendez et al.,
1999; Sathar et al., 2004] raising the possibility that infants may acquire GBV-C
horizontally from someone other than the mother post-delivery. Although it is not known
if GBV-C can be transmitted postnatally from mother to child or between unrelated
persons through routine contact, this is thought to be unlikely [Feucht et al., 1999; Wejstal et al., 1999]. Feucht et al. followed 58 children, 9 of whom had GBV-C-infected mothers, and none became GBV-C infected over a three-year period [Feucht et al., 1999]. Westjal also observed that no infants of 53 GBV-C negative mothers acquired GBV-C after one to two years of follow-up, concluding that this provides a strong argument against other common source of infection in early childhood. In another study, the negative twin of a GBV-C infected infant remained negative despite the presence of salivary GBV-C in the mother and twin sibling [Fischler et al., 1997]. GBV-C RNA has not been isolated in breast milk [Schroter et al., 2000] and is not thought to be transmitted through breastfeeding. However, the possibility that some GBV-C is acquired by the infant following delivery from maternal saliva or elsewhere cannot be ruled out. Some investigators have suggested that horizontal transmission of GBV-C genotype 1 is more common than vertical transmission based on the low observed prevalence of GBV-C genotype 1 RNA in cord blood samples and increased prevalence of GBV-C RNA (prior to transfusion) among children aged 3 years and older admitted with malaria related anemia, compared to those younger than 3 years of age [Li et al., 2006b]. This conclusion, however, may not be valid, as the cord blood from infants ultimately found to be HIV-infected infants is often HIV RNA negative even though MTCT of HIV has occurred [Biggar et al., 1997], and it is unclear if the GBV-C-infected older children had had previous hospitalizations and transfusions which might explain their infections. Still, it is interesting to note that the only studies with evidence of horizontal transmission [Li et al., 2006b; Menendez et al., 1999; Sathar et al., 2004] were all conducted in Africa indicating, perhaps as Li suggested, differences in transmission patterns related to
African specific genotypes or behavioural practices. On the other hand, sample contamination or imperfect sensitivity and specificity of the PCR test may have been responsible for these results.

### 1.2.6 VERTICAL TRANSMISSION OF HIV

Vertical HIV-1 transmission is now highly preventable and rates of approximately 21% in U.S. non-breast feeding cohorts in the early 1990s have decreased to approximately 1.5% with standard ARV combination therapy [Bulterys, 2000]. However, many children in poorer parts of the world still become infected each year due to lack of antenatal HIV testing and prophylaxis. Identified risk factors for perinatal HIV-1 transmission include high maternal HIV viral load, vaginal delivery, advanced maternal disease, low CD4+ count or percent, duration of membrane rupture greater than 4 hours, premature delivery (<37 weeks), viral genotype/phenotype, and breastfeeding [Bulterys, 2000]. Other factors for which evidence is not conclusive include genetic factors, immature immune system in the infant, viral strain diversity, maternal neutralizing antibody, illicit drug use during pregnancy, frequency of unprotected sexual intercourse, multiple sex partners during pregnancy, maternal nutritional status, anemia during pregnancy, cigarette smoking, chorioamnionitis, abruptio placenta, syphilis and other STIs, fetal scalp electrodes, episiotomy and vaginal tears [Bulterys, 2000]. Maternal HIV-1 RNA level, especially near the time of delivery, is most strongly correlated with MTCT-HIV [Bulterys, 2000; Shaffer et al., 1999]. However, there does not appear to be a threshold under which transmission does not occur nor a threshold over
which transmission always occurs. Only 1% of ARV-treated women with RNA<1000 copies/ml transmitted HIV to their infant compared to 9.8% of untreated women [Ioannidis et al., 2001]. Antiretroviral treatment reduces maternal HIV viral load but has also been found protective above and beyond its effect on viral load and appears to independently reduce the risk of transmission at all levels of viral load, even in women with RNA<1,000 copies [Bultery, 2000; Sperling et al., 1996]. Recent studies have also reported that female infants are more likely to become HIV-infected but the biological basis for this is unknown [Biggar et al., 2006; Galli et al., 2005; Taha et al., 2005].

In ARV-untreated women, the approximate timing of transmission is estimated to be 5% prior to 36 weeks, 20% from 36 weeks through delivery and a further 15% during breastfeeding [Kourtis et al., 2001]. Therefore, 80% of infections in non-breast fed cohorts occur either late in gestation or during delivery [Kourtis et al., 2001].

1.2.7 VERTICAL TRANSMISSION OF HCV

HCV is transmitted vertically to only approximately 5% of infants of infected mothers [Herrine, 2006]. HCV is thought to be transmitted in utero in approximately 33% (95% CI, 19% to 46%) of infected children based on the early detection of HCV RNA during the first three days of life [Mok et al., 2005]. High HCV maternal viral load has been associated with HCV vertical transmission in some but not all studies [Ceci et al., 2001; Herrine, 2006]. While prolonged duration of rupture of membranes has been associated with increased transmission, caesarean section has not been found to be
protection in observational studies [Herrine, 2006]. Like GBV-C, its closest relative among the flaviviruses, HCV has not been found in breast-milk [Kage et al., 1997]. HCV RNA is detectable in saliva in some carrier mothers (~36%) but does not appear to be easily transmitted to the infant and postpartum transmission, if it occurs at all, is rare [Kage et al., 1997]. HCV transmission, like HIV transmission, has been observed more often among female infants in one study [European Pediatric Hepatitis C Virus Network, 2005].

1.2.8 THE BENEFICIAL EFFECT OF GBV-C COINFECTION ON HIV PROGRESSION– EPIDEMIOLOGIC EVIDENCE

Although it was initially suspected that, like hepatitis B and C viruses, GBV-C might accelerate the course of HIV disease, two of the first studies examining GBV-C and HIV coinfection observed that GBV-C is not harmful and, in fact, may be beneficial to coinfected persons [Heringlake et al., 1998; Toyoda et al., 1998], while a third did not [Sabin et al., 1998]. Toyoda et al., in a small study of 41 Japanese hemophilia patients who became HIV infected in the early 1980’s, including 11 who had been GBV-C infected for a period of at least five years, reported that mean HIV RNA levels were much lower in GBV-C infected (35,000 viral copies/ml) compared to GBV-C-uninfected patients (58,000 viral copies/ml) and that there was slower progression to AIDS and death, although these differences were not statistically significant [Toyoda et al., 1998]. Heringlake et al. investigated a larger cohort of 197 patients in Germany who had been infected with HIV for an approximately equal length of time. CD4+ counts were
significantly higher in GBV-C-viremic (344 cells/ul) versus GBV-C antibody-positive (259 cells/ul) and GBV-C-negative (170 cells/ul) patients and mean survival among GBV-C viremic patients was significantly longer (1,052 days) compared to both GBV-C antibody positive (930 days) and GBV-C negative (mean 777 days) patients. The improvement in the antibody-positive group’s survival compared to the GBV-C negative group was also significant [Heringlake et al., 1998].

Since then, eight additional studies have reported a beneficial effect on survival of GBV-C viremia and/or antibody [Aboulker et al., 2003; Lefrere et al., 1999c; Nunnari et al., 2003; Tillmann et al., 2001; Williams et al., 2004; Xiang et al., 2001; Yeo et al., 2000; Yirrell et al., 2007] while four have reported no difference [Birk et al., 2002; Kaye et al., 2005; Ryt Hansen et al., 2006]. Others have had different results for baseline and persistent GBV-C and are difficult to classify [Bjorkman et al., 2004; Van der Bij et al., 2005].

The GBV-C studies are difficult to compare as some investigators analysed subjects with GBV-C RNA and antibody together, while others did not test for GBV-C antibody, effectively classifying those with past infection together with those never infected. If, as Heringlake observed, the beneficial effect of presence of GBV-C antibody is mid-way between presence of GBV-C RNA and never having been exposed to GBV-C infection (i.e. GBV-C RNA-negative and antibody-negative), either method of classification of the GBV-C antibody group could nullify a real effect. Other problems include small sample sizes, unknown duration of HIV and GBV-C infection, and variable
follow-up times. Some studies analyzed early HIV infection and some later infection. As length of HIV infection and persistence of GBV-C infection are associated with survival, this is problematic. Ideally, a well designed GBV-C study would have a large sample size, be longitudinal with known durations of HIV and GBV-C infection, test for both RNA and antibody, and control for HIV viral load, CD4+ count, and ART.

Lefrere et al. studied 95 HIV-infected patients in France who were followed for a mean of 8.5 years [Lefrere et al., 1999c]. The 23 persons who were GBV-C viremic for a mean period of 7.7 years had improved CD4+ counts, HIV RNA load and survival compared to GBV-C RNA negative subjects. Statistically significant differences in mean HIV RNA load and CD4+ count were not observed until the 3rd and 5th year of follow-up respectively, suggesting that prolonged carriage of the virus may be required for a beneficial effect. Yeo et al. found that GBV-C viremic and GBV-C antibody-positive patients had significantly improved CD4+ counts and 12 year AIDS free survival (HR 0.59) adjusted for HIV RNA load, CD4+ and CD8+ cell count, and CC chemokine receptor 5 (CCR5) genotype compared to GBV-C negative patients, but did not have lower HIV plasma viral loads [Yeo et al., 2000]. Xiang et al., in one of the largest studies of GBV-C and HIV coinfection with 362 HIV-infected patients, reported that mortality in patients without GBV-C RNA compared to those with GBV-C RNA was significantly higher (56.4% vs. 28.5%) over a mean follow-up period of 4.1 years, with a relative risk of 3.7 (95% CI, 2.5-5.4) adjusted for treatment, baseline CD4+ count, age, sex, race and mode of HIV transmission [Xiang et al., 2001]. Similarly, Tillman et al., in an extension of the Heringlake study, found improved survival from both date of first positive HIV test
and date of GBV-C testing and reported that progression to AIDS from both start dates was significantly slower (p<0.001 and p<0.002, respectively) among GBV-C-infected compared to GBV-C-antibody-positive and GBV-C-uninfected persons together [Tillmann et al., 2001]. Even after the development of AIDS, the presence of GBV-C RNA was associated with a better prognosis (p=0.007). Tillman et al. also reported a significant inverse correlation between HIV plasma viral load and GBV-C plasma viral load even when controlling for CD4+ count and HAART. Nunnari et al., in an analysis of 80 asymptomatic HIV-1-infected patients followed for over eight years, reported improved AIDS free survival, CD4+ count and HIV RNA load in GBV-C infected persons, as well as an improved response to HAART [Nunnari et al., 2003]. Other studies have reported significant associations between GBV-C viremia and reduced HIV viral load and/or increased CD4+ cell count [Addo et al., 2004; Custer et al., 2004; Hattori et al., 2007; Li et al., 2006a]. Canducci et al. also observed that GBV-C viremia was more common among long-term non-progressors (i.e. those with CD4+ counts ≥ 500 cells/ul) than among those with CD4+ counts less than 200 cells/ul (38% vs. 20%; OR 2.5, p=0.064) [Canducci et al., 2003]. GBV-C genotype may be an important determinant of a beneficial effect of GBV-C on CD4+ count as coinfection with GBV-C genotype 2 was associated with significantly higher CD4+ cell counts compared to genotype 1 in one study [Schwarze-Zander et al., 2006].

Despite these positive studies, the existence and significance of a beneficial effect of GBV-C on HIV progression remains controversial. Sabin et al., in an early study of 94 HIV-HCV coinfected hemophilia patients in England, found faster progression to AIDS
and death among those with GBV-C exposure (GBV-C-RNA or -antibody positive) compared to those without markers for exposure (GBV-C-RNA and -antibody negative) but this was not statistically significant [Sabin et al., 1998]. Birk et al. observed no difference between those with GBV-C RNA compared to those without among 157 HIV-infected patients in Sweden followed for a median of seven years until 1995 (pre-HAART) in time to CD4+ count < 200 cell/ul, AIDS, or death [Birk et al., 2002]. A recently published Danish study also found no difference in time to AIDS or death among 112 HIV-infected men with and without GBV-C RNA followed for a mean of 6.7 years in the pre-HAART era [Ryt Hansen et al., 2006]. An unpublished study of 180 HIV-infected patients with early HIV disease also found worse survival in those with GBV-C infection (definition of infection and statistical significance not presented) [Brust et al., 2002]. An African study also observed no difference in mortality, CD4+ cell percent or HIV viral load between women with and without GBV-C RNA infected with HIV-1 and HIV-2 followed for a median time of 6 years [Kaye et al., 2005]. Variation in the duration and order of HIV and GBV-C infection across these studies as well as the definition of infection may be responsible for the discrepant results.

The interaction between GBV-C and HIV was examined in patients receiving antiretroviral therapy in several studies to determine if GBV-C coinfected patients had a more favourable response to therapy than patients infected with HIV alone. Some studies examining response to therapy observed a beneficial effect and some did not. None reported a beneficial effect of GBV-C infection on survival for persons receiving HAART. An unpublished study from France of 326 HIV-infected patients receiving
antiretroviral therapy but not HAART reported slower disease progression and greater CD4+ cell response to therapy, but no difference in reduction of HIV RNA, adjusted for baseline CD4+ count, baseline HIV RNA, and sex, among GBV-C viremic patients compared to non-viremic patients [Aboulker et al., 2003]. Rodriguez et al. observed that GBV-C viremia was associated with virologic HIV treatment success in univariate (GBV-C RNA 46%, GBV-C antibody 37%, GBV-C negative 23%) and multivariate analysis (AOR 3.07, 95%CI 1.9-7.75) adjusted for baseline CD4+ count and plasma HIV RNA. They also reported significantly higher baseline CD4+ count (60 vs. 30 cells/uL, p=0.043) and CD8+ counts (700 vs. 610 cells/uL, p=0.048) and significantly lower plasma HIV RNA levels (5.11 vs. 5.29 log_{10} copies, p=0.05) with presence and absence of GBV-C RNA, respectively, in 146 HIV-infected patients on HAART [Rodriguez et al., 2003]. Souza et al. examined 175 HIV-infected patients in Brazil and also reported improved response to HAART therapy, with GBV-C coinfected patients experiencing an approximately 0.5 log_{10} greater decrease in HIV RNA load compared to GBV-C uninfected patients [Souza et al., 2006]. Conversely, Brumme et al. examined 441 patients initiating antiretroviral therapy from 1996 to 1998 (HAART era) and observed that, although baseline presence of GBV-C RNA was associated with a lower HIV RNA load, baseline GBV-C RNA status was not associated with time to virologic success or failure or time to immunologic failure [Brumme et al., 2002]. Sheng et al. also observed that patients with persistent GBV-C viremia had lower baseline plasma HIV RNA load but no improvement in immunologic, virologic, or clinical response to HAART [Sheng et al., 2007]. Antonucci et al. found that, while there was no difference in time to virologic suppression or CD4+ response between GBV-C viremic and non-viremic patients
initiating therapy with HAART, GBV-C viremic patients had a lower risk of HIV rebound (HR 0.56, 95% CI 0.34-0.93) [Antonucci et al., 2005].

Due to the low death rate among HIV-infected patients on HAART, survival may be a difficult outcome to analyze in these cohorts. Restricting their analysis to the period when persons received HAART (1996 to 2000), Tillman et al. reported that GBV-C RNA-positive persons had 96% survival, anti-E2-positive persons 70% and those GBV-C negative 60% survival (p=0.01) but this was not significant in multivariate analysis [Tillmann et al., 2001]. Williams et al. reported no difference in survival among 352 GBV-C-viremic and GBV-C non-viremic women receiving HAART [Williams et al., 2005]. Quiros-Roldan et al. also reported no difference in progression to AIDS or survival among 285 HIV-infected patients on HAART; the proportion of deaths in both GBV-C viremic and non-viremic patients was 1.0% over 5 years [Quiros-Roldan et al., 2002]. However, ART remains an interesting factor that may play a role in the interaction between HIV and GBV-C. A recent study observed that HAART initiation resulted in decreased HIV replication and increased GBV-C replication [Bjorkman et al., 2007].

Four recent studies examined the interaction between GBV-C and HIV longitudinally. All studies observed that loss of GBV-C RNA was associated with significantly worse survival [Bjorkman et al., 2004; Van der Bij et al., 2005; Williams et al., 2004; Yirrell et al., 2007]. Persistent coinfection with GBV-C virus was associated with improved survival compared to those persistently negative for GBV-C RNA in HIV-
infected adults in three of the studies [Van der Bij et al., 2005; Williams et al., 2004; Yirrell et al., 2007]. The fourth study found a beneficial effect of GBV-C viremia at baseline on survival only in the subgroup with more advanced HIV disease and no beneficial effect of persistent GBV-C infection [Bjorkman et al., 2004]. Williams et al., in a study of incident HIV infections among men from the Multicenter Acquired Immunodeficiency Syndrome Cohort Study (MACS), evaluated GBV-C status at 12-18 months and 5-6 years following HIV-seroconversion in 138 men [Williams et al., 2004]. They observed that, while GBV-C status was not associated with survival at baseline, it was protective 5-6 years later and that those without GBV-C RNA were 2.8 times more likely to die (95% CI, 1.3-5.8) than those with GBV-C RNA. Compared to men who were GBV-C RNA positive at both visits, men who were GBV-C RNA negative at both visits were 2.4 (GBV-C-antibody positive) and 2.6 (GBV-C-antibody negative) times more likely to die. The worst prognosis was for men who were GBV-C RNA positive at the first visit and had lost GBV-C RNA over the course of the study; they were 5.9 times more likely to die than those with persistent GBV-C RNA (95% CI 2.2-15.4). Nine of twelve men who cleared GBV-C RNA were noted not to have GBV-C antibodies in their plasma at the later visit and eight subsequently died [Stapleton et al., 2004]. The effect of GBV-C RNA was independent of CD4+ count and HIV plasma viral load at baseline but not at 5-6 years following seroconversion, suggesting that GBV-C RNA was associated with changes in these variables. Bjorkman et al. followed 163 patients from within two years following HIV diagnosis (date of seroconversion unknown) to initiation of HAART, death, or last visit (median time 4.3 years); some were followed for up to 18 years. GBV-C RNA was significantly less prevalent in patients with AIDS at
recruitment. A significant improvement in survival for patients GBV-C viremic at baseline was found among a subgroup of patients with symptomatic disease (CDC stage B or C, or CD4+ count < 300 cells/ul). There was no reported difference in survival between those persistently positive and those persistently negative for GBV-C RNA [Bjorkman et al., 2004], although Kaplan-Meier analysis appears to show an overall beneficial effect in all cause mortality at approximately 4-6 years of follow-up. However, those who lost GBV-C RNA without detectable antibody (11/14 who lost GBV-C RNA) had significantly worse survival than those persistently positive, persistently negative or those who acquired GBV-C over the study period (p=0.018, anti-E2 positive patients excluded). Van der Bij et al. evaluated 326 homosexual men within an estimated two years following seroconversion (exact date unknown for 62% of the cohort) and at study end after a median follow-up time of eight years [Van der Bij et al., 2005]. CD4+ counts were initially worse among those with GBV-C RNA and baseline GBV-C viremia was associated with increased risk of HIV progression and death in univariate analysis. However, persistent GBV-C RNA was strongly associated with improved survival compared to those without GBV-C RNA at both study visits in univariate and several multivariate models (OR 0.52-0.57) adjusting for age at seroconversion, HAART, CCR5 genotype, CD4+ count, CD4+ count one year after seroconversion, log_{10} HIV RNA, and log_{10} HIV RNA as a time-dependent covariate, but was not significant after adjusting for CD4+ count as a time dependent covariate. This is similar to the results reported by Williams et al., where the observed beneficial effect of GBV-C viremia on survival disappeared after adjustment for late visit CD4+ count. Despite interpretation by Van der Bij to the contrary, this does not necessarily mean that
GBV-C is not associated with survival, as CD4+ cell count may mediate the effect of GBV-C on survival. Loss of GBV-C viremia was also significantly associated with increased risk of death (HR 3.3-2.2) until adjusted for CD4+ count as a time dependent covariate. Loss of viremia occurred in 78/137 men and only 14 (18%) of them tested positive for GBV-C antibody by study end. Similar results were reported recently by Yirrell et al. in a study of HIV-infected men and women in rural Uganda [Yirrell et al., 2007]. Persistent GBV-C was associated with a slower decline in CD4+ cells and longer survival and those who cleared GBV-C had the worst survival.

If there is an effect of GBV-C on HIV progression, it appears to occur late in HIV disease progression. A recent meta-analysis synthesized the results from six studies and found that GBV-C status measured within two years of HIV seroconversion had no effect on survival while GBV-C measured two years or more post-seroconversion was associated with a reduced hazard of mortality [Zhang et al., 2006]. Thus, the association between GBV-C coinfection and survival (the hazard ratio) appears to change over time with duration of HIV and GBV-C infection.

The literature contains conflicting results regarding the potential beneficial effect of GBV-C. Characteristics of a well-designed epidemiologic study to determine the effects of GBV-C include a large sample size, a longitudinal study design with known durations of HIV and GBV-C infection, measurement of both HIV progression and GBV-C status over time and identification of both GBV-C RNA and antibody. Based on these criteria, the longitudinal studies by Williams et al. and Van der Bij et al. provide the
strongest epidemiologic evidence. However, neither study is without limitations. A strength of the study by Williams et al. was that it was the only study to use incident HIV infections while a weakness was its smaller sample size. Van der Bij had a very large study sample but was only able to estimate the date of HIV seroconversion of his subjects within 2 years. The findings of the Van der Bij and Williams studies were similar and the analyses were adjusted for similar potential confounders, yet the conclusions regarding the directionality of the effect were opposite. Van der Bij et al. concluded that because the beneficial effect of GBV-C disappeared after adjustment for time varying CD4+ count, that presence of GBV-C was likely a function of CD4+ cell count, while Williams et al. concluded that GBV-C was beneficial and associated with changes in CD4+ count and HIV viral load. The lack of agreement in the results of studies in the literature provided further impetus for our work.

1.2.9 POSSIBLE BIOLOGIC MECHANISMS FOR THE INTERACTION BETWEEN GBV-C AND HIV

Several biologically plausible mechanisms have been proposed to explain the observed interaction between HIV and GBV-C (see Figure 2 below). Some mechanisms supporting the theory that GBV-C has an inhibitory effect on HIV replication include: i) GBV-C infection directly inhibits HIV replication [Xiang et al., 2003]; ii) GBV-C infection increases production of chemokine ligands for the CC chemokine receptor 5 (CCR5) blocking HIV cell entry [Nattermann et al., 2003; Xiang et al., 2004]; iii) GBV-C
infection down regulates the CCR5 receptor [Nattermann et al., 2003; Xiang et al., 2004]; and iv) GBV-C infection modulates the T-helper (TH) cytokines [Nunnari et al., 2003]. These four potential mechanisms are discussed in more detail in the following sections.

1.2.9.1 Interference with viral replication

_In vivo_ and _in vitro_ evidence supports the belief that GBV-C may interfere with HIV replication, possibly directly. Several epidemiologic studies have observed reduced HIV viral load in persons with GBV-C RNA compared to those without [Brumme et al., 2002; Hattori et al., 2007; Li et al., 2006a; Rodriguez et al., 2003; Tillmann et al., 2001; Toyoda et al., 1998] and some have observed an inverse correlation between GBV-C viral load and HIV viral load, although this was often fairly weak [Barnes et al., 2007; Hattori et al., 2007; Li et al., 2006a; Tillmann et al., 2001]. _In vitro_ studies have also demonstrated reduced HIV replication in cell cultures coinfected with GBV-C [Jung et al., 2005; Xiang et al., 2001]. In one study [Xiang et al., 2001], cells simultaneously infected with GBV-C and HIV experienced a 49% decrease in HIV replication 6 days post infection as measured by p24 antigen in supernatant fluid, while cell cultures infected with GBV-C first experienced a 99% reduction in HIV replication six days following HIV infection [Xiang et al., 2001]. However, GBV-C in cell culture did not prevent entry of HIV into cells [Xiang et al., 2001]. There is some evidence that the GBV-C glycoprotein E2 may interfere with the early steps of HIV replication such as binding or membrane fusion but not the later steps between entry and particle assembly [Jung et al., 2007]. GBV-C has been found to inhibit HIV strains that use both the
CXCR4 and CCR5 coreceptor [Jung et al., 2005; Xiang et al., 2003] and GBV-C replication appears to be required for inhibition [Xiang et al., 2003]. However, because GBV-C infects only one in 5,000 cells and transcriptionally active HIV is found in only one in every 100-400 CD4+ cells, the marked suppression seen in vitro may not be reproducible in vivo [Xiang et al., 2004].

1.2.9.2 Induction of HIV-inhibitory chemokines

One possible mechanism by which GBV-C may inhibit HIV is through the induction of HIV-inhibitory chemokines. Chemokine receptors play an important role in the transmission and pathogenesis of HIV. To infect a target cell, the HIV envelope glycoprotein ‘gp 120’ must interact with both the CD4 cellular receptor and an HIV-coreceptor on the CD4+ cell. The two major HIV coreceptors are CCR5 and CXCR4 that bind with ‘R-5’ and ‘R-4’ strains of HIV-1, respectively [Verani and Lusso, 2002]. However, there may be other minor chemokine receptors that bind with other strains [Zaitseva et al., 2003]. Most primary HIV strains use CCR5 as a coreceptor and therefore are sensitive to inhibition by CCR5-ligand chemokines (RANTES, MIP-1α, MIP-1β) which block the receptor, preventing the HIV virion from attaching and entering the cell [Verani and Lusso, 2002]. The crucial role of CCR5 is confirmed by the almost complete resistance to HIV in people carrying a homozygous 32 bp deletion within the CCR5 gene (CCR5-delta32) [Verani and Lusso, 2002]. CCR5 also appears to be important for mother-to-child transmission of HIV, as R-5 specific viral load has been found to be more strongly correlated with transmission than total viral load [Burger et al., 2003].
small proportion of HIV isolates which usually appear with progression to AIDS use CXCR4 as a coreceptor and are inhibited by the CXCR4-ligand SDF-1.

In vitro, GBV-C-infected cells were found in one study to increase secretions of the CCR5 chemokines RANTES, MIP-1α, MIP-1β and the CXCR4 chemokine SDF-1 compared to mock-infected cells [Xiang et al., 2004]. Nattermann et al. also demonstrated in laboratory stimulation studies that the binding of GBV-C envelope protein E2 to CD81 on T lymphocytes induced a dose-dependent release of RANTES and down-regulation of the CCR5 coreceptor in vitro [Nattermann et al., 2003]. However, Jung et al. stimulated cells with the GBV-C glycoprotein E2 protein and observed decreased HIV replication but no increase in RANTES, MIP-1α, MIP-1β, nor SDF-1 in the supernatant fluid, concluding that that the mechanism of the GBV-C E2-mediated HIV entry impairment still needs to be clarified. Jung et al. also observed no downregulation of the CXCR4 coreceptor or its respective chemokine, SDF-1 in GBV-C infected cell cultures [Jung et al., 2005].

Evidence from in vivo studies has not supported an effect of GBV-C on cytokine or chemokine expression to date. Hattori et al., in a study of 182 HIV-infected patients, observed significantly lower levels of RANTES in the blood of GBV-C-coinfected persons compared to GBV-C-uninfected persons, although the levels of RANTES increased in GBV-C-infected persons and decreased in GBV-C-uninfected persons over one year of follow-up [Hattori et al., 2007]. The authors concluded that upregulated RANTES is not responsible for decreased HIV replication and that another mechanism
associated with GBV-C infection must be responsible for the beneficial effect of GBV-C observed in the study [Hattori et al., 2007]. In another study of HIV-infected patients, no difference was seen in cytokines SDF-1, CCL5, IL-7, or TNF-α between GBV-C coinfectected and GBV-C-uninfected patients [Gimenez-Barcons et al., 2005].

1.2.9.3 Down-regulation of the CCR5 coreceptor

The level of CCR5 expression is an important determinant for HIV transmission and disease progression [Nattermann et al., 2003]. Nattermann et al. observed that CCR5 expression was reduced on CD4+ and CD8+ lymphocytes of GBV-C infected subjects by 53% and 36%, respectively, in cross-sectional analysis and suggested that a RANTES mediated effect on CCR5 expression may explain delayed HIV progression in patients coinfectected with GBV-C [Nattermann et al., 2003]. Xiang et al. observed reduced expression of the HIV coreceptor CCR5 in GBV-C infected cells after three days [Xiang et al., 2004] but no difference in surface expression of CD4, CCR5 and CXCR4 during the first 24 hours [Xiang et al., 2001]. However, Brumme et al. found no correlation between detection of GBV-C RNA and polymorphisms in chemokine receptors and concluded that ‘any impact of GBV-C viremia is unlikely to be linked to the HIV co-receptor mutation’ [Brumme et al., 2002].

1.2.9.4 Maintaining a TH-1 cytokine profile

As HIV disease progresses to AIDS, an individual’s immune profile often changes from what is known as T helper type 1 (Th1) immunity to T helper type 2 (Th2) immunity. Th1 immunity is characterized by intense phagocytic activity and TH-1
lymphocytes secrete interleukin (IL-2), interferon-γ and lymphotoxin-α [Spellberg and Edwards, 2001]. Th2 immunity is characterized by high antibody titres and secretion of IL-4, IL-5, IL-9, IL-10 and IL-13 [Spellberg and Edwards, 2001]. Progression to AIDS is correlated with the inability of mononuclear cells to produce IL-2 and IL-12 which stimulate Th1 activity and interferon-γ, and with increased production of IL-4 and IL-10 [Nunnari et al., 2003; Spellberg and Edwards, 2001]. Nunnari et al. followed HIV-infected individuals for over eight years and observed that GBV-C RNA-negative persons switched to a predominant Th2 response while GBV-C RNA-positive persons maintained stable Th1 cytokine levels over the follow-up period. Interleukin (IL)-2 and IL-12 declined by 85% and 83% and IL-4 and IL-10 increased by 654% and 395% in GBV-C RNA-negative persons. However it is unclear if this is a cause or a consequence of HIV disease progression [Nunnari et al., 2003].

Figure 2 below presents a model of the various mechanisms by which GBV-C might influence the replication of HIV and delay the development of AIDS. However, the true nature of the interaction remains unknown and a causal relation between GBV-C coinfection and prolonged survival of persons with HIV infection cannot be assumed.
1.2.9.5 GBV-C infection as a consequence of HIV progression

While some investigators [Stapleton et al., 2004; Williams et al., 2004] have posited that GBV-C infection may improve survival over the long term due to one or more of the biologically plausible mechanisms mentioned above, others [Bjorkman et al., 2004; Van der Bij et al., 2005] suggest that causation may be reversed and that GBV-C infection status is likely secondary to HIV progression. As CD4+ cells are a site of replication for GBV-C and multivariate models imply some association between GBV-C...
RNA and time varying CD4+ count, loss of CD4+ cells with HIV progression has been suggested as the reason for absence of GBV-C RNA in those with worse progression [Van der Bij et al., 2005]. A significant beneficial association between GBV-C infection and CD4+ cell count has been observed in several studies [Aboulker et al., 2003; Handelsman et al., 2007; Heringlake et al., 1998; Lefrere et al., 1999c; Rodriguez et al., 2003; Yeo et al., 2000]; however, the direction of the effect is unknown. Arguing against the CD4+ hypothesis is the fact that CD4+ cell depletion (CD4+ cell count <200 cell/ul) was observed in 39% of GBV-C coinfected patients (including 17% with less than 50 cells/ul) and that GBV-C RNA had a beneficial effect on survival at all levels of CD4+ cell count in one study [Xiang et al., 2001]. In addition, because GBV-C has also been shown to replicate in B cells as well as CD8+ T lymphocytes, CD4+ cell depletion cannot be the sole reason for clearance of GBV-C [George et al., 2006]. However, the recent finding that GBV-C replication was enhanced and HIV replication diminished with the initiation of HAART, suggests that GBV-C replication may be dependent on something related to HIV replication [Bjorkman et al., 2007].

1.2.10 ASSOCIATION OF MATERNAL AND INFANT GBV-C INFECTION WITH PREVENTION OF MTCT OF HIV

Because GBV-C has been associated with decreased HIV viral load, the principal risk factor for vertical and horizontal HIV-1 transmission [Bulteryys, 2000; Fideli et al., 2001; Quinn et al., 2000; Shaffer et al., 1999], GBV-C itself or an associated mechanism may be able to reduce HIV transmission. When we began the present study, no studies
had been published on the association between GBV-C infection and HIV acquisition; however, since that time, the results of several studies have been reported.

Bisson et al. examined the association between GBV-C viremia and susceptibility to HIV infection in adults and reported that GBV-C viremia prior to HIV infection did not appear to protect against HIV acquisition (OR 1.3, 95% CI 0.68 – 2.6) [Bisson et al., 2005]. Two studies examined the association between maternal GBV-C coinfection and reduced MTCT of HIV and did not observe a significant association [Handelsman et al., 2007; Weintrob et al., 2004]. Handelsman et al. examined 397 mother-infant pairs from the Women and Infants Transmission Study and observed that maternal GBV-C viremia was not significantly associated with decreased MTCT of HIV (OR 0.79, p=0.26) except possibly among among GBV-C viremic women on HAART (OR 0.30, p=0.06) [Handelsman et al., 2007]. Weintrob et al. studied 128 ART-naïve mother-infant pairs in Tanzania and also did not observe a significant difference in MTCT of HIV between women with GBV-C RNA (7/25, 28%), women with GBV-C antibody (6/26, 23%), and women with no GBV-C RNA or antibody (20/77, 26%) [Weintrob et al., 2004]. Two other studies were too small to draw definitive conclusions. Barqasho et al. studied 52 ART-naïve mother-infant pairs in Sweden. None of the three mothers with GBV-C viremia transmitted HIV to their infant, compared to 4/14 (29%) mothers with GBV-C antibody and 6/29 (21%) mothers without GBV-C RNA or antibody [Barqasho et al., 2004]. Sather et al. studied 20 ART-naïve mother-infant pairs in South Africa. Two of five mothers with GBV-C viremia (40%) transmitted HIV to their infants compared to one of 15 (6.7%) GBV-C RNA-negative mothers [Sathar et al., 2004].
No studies have yet been conducted to determine whether infant GBV-C infection is associated with MTCT of HIV. However, three small studies of MTCT of GBV-C among HIV-coinfected women, with from two to nine GBV-C infected infants, did not observe an association with MTCT of HIV. In these studies, rates of HIV-infection among GBV-C infected children compared to GBV-C uninfected children were: 1/5 (20%) compared to 10/58 (19%); 1/9 (11%), compared to 0/20 (0%), and 1/2 (50%) compared to 1/3 (33%) [de Martino et al., 1998; Palomba et al., 1999; Sathar et al., 2004].

1.2.11 VIRAL COINFECTION

Several diseases have been observed to prevent or decrease the severity of other diseases and, in the past, such observations have led to important therapeutic advances such as the use of cowpox to prevent smallpox [Stiehm, 2006]. Several viruses in addition to GBV-C may have the potential to inhibit HIV viral replication including HTLV-II which like GBV-C, has been associated with slower progression of HIV and increased CD4+ count [Turci, 2005]. HIV downregulation has also been observed in cell cultures by HTLV-II and dengue virus, and in vivo by scrub typhus and measles [Kannangara et al., 2005; McLinden et al., 2008]. Conversely, other persistent viral infections such as cytomegalovirus (CMV), hepatitis C, and herpes simplex virus (HSV-1
and HSV-2) [Northfield et al., 2005; Pomerantz and Nunnari, 2004] or helminth infections [Modjarrad, 2009] may augment the transmission and/or progression of HIV.

There are few studies of maternal coinfections and vertical transmission; however vertical transmission of HCV is more common among HIV coinfected women, and vertical transmission of HIV also appears to be more common among HCV coinfected women [England et al., 2006]. Contrary to expectation, chorioamnionitis was associated with reduced MTCT of HIV in one study [Schwartz et al., 2000] and neither polymorphonuclear cell nor mononuclear placental infiltrations were associated with mother-to-child transmission of HIV in another [Goldenberg et al., 2006]. Placental malaria has been associated with both increased and decreased MTCT of HIV in separate studies, possibly depending on the density of the malaria [Ayisi et al., 2004; Naniche et al., 2008].

1.3 JUSTIFICATION FOR STUDY

Our study is the largest study to date to examine the relationship between maternal GBV-C infection and MTCT of HIV and the first study to address the association between perinatally acquired GBV-C and MTCT of HIV. Better understanding of the interaction between HIV and GBV-C in the perinatal context may shed light on the possible beneficial effect of GBV-C and help elucidate useful biologic pathways for the prevention and treatment of HIV.
1.4 OBJECTIVES

1. To determine if maternal or infant GBV-C infection is associated with a reduced rate of mother-to-child HIV transmission.

2. To examine the rate and correlates of GBV-C mother-to-child transmission among HIV-infected mothers, including maternal, infant and delivery characteristics.

3. To determine the prevalence and correlates of GBV-C infection among pregnant women in Thailand, including HIV status and biologic, demographic and behavioural characteristics.

1.5 ORGANIZATION OF THE THESIS

The remainder of this paper-based thesis is organized as follows. Chapter 2 describes the study population and methods used for the study. Chapters 3 to 5 are the manuscripts for Objectives 1 to 3 in that order. Chapter 3 was published in the Journal of Infectious Diseases [Bhanich Supapol et al., 2008] with an editorial commentary [Bjorkman and Widell, 2008]. Chapter 4 was also recently published in the Journal of
Infectious Diseases [Bhanich Supapol et al., 2009]. Chapter 5 is in the clearance process at CDC. Given the international nature of the study and that it involved existing high profile studies, there are several study collaborators and coauthors for publication. The author’s contribution to the study is detailed in Appendix 1. Population trees describing sample availability and the GBV-C test results for women and infants in each of the studies are found in Appendix 2. A report of the laboratory validation study we conducted prior to commencing the GBV-C study is in Appendix 3. Appendix 4 contains some results that were not presented in the manuscripts but that are referred to in the discussion.
1.6 REFERENCES


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Chapter 2

Methods

2.1 STUDY POPULATION AND DESIGN

2.1.1 STUDY DESIGN

This study used data and stored blood collected during the course of three perinatal HIV transmission studies (hereafter referred to as Peri-1, Peri-2, and Peri-3) conducted jointly by the Thailand Ministry of Public Health and the U.S. Centers for Disease Control and Prevention (TUC) at Rajawithi and Siriraj hospitals, two large public hospitals in Bangkok from 1992 to 2004. These hospitals initiated antenatal HIV testing and counselling in 1992 [Siriwasin et al., 1998] and HIV-infected women were discouraged from breastfeeding. All women participating in the original studies who consented to have their blood stored for future research and who had a delivery or third trimester blood specimen available for testing as of March 31, 2004 were included in the present study. We were not permitted to use the final stored blood specimen from mothers or infants for the present study. Maternal specimens were tested for GBV-C RNA and GBV-C antibody and those testing RNA-positive were also tested for GBV-C genotype. The maternal delivery specimen was preferred as we wished to determine the
mother’s GBV-C status at the time of delivery, the time when most mother-to-child-transmission (MTCT) of HIV occurs [Kourtis et al., 2001], in relation to our primary objective. Infants of GBV-C viremic mothers with a four- or six-month plasma specimen (the time during which GBV-C RNA has been detectable in GBV-C-infected infants in other studies) [Viazov et al., 1997; Zanetti et al., 1998; Zuin et al., 1999] were tested for GBV-C RNA. If the four- (Peri-3) or six-month (Peri-1 and 2) infant plasma specimen was not available, specimens from other times were tested. Peri-1 infants whose initial specimen tested GBV-C-RNA-positive were also tested at other times to help determine the timing of GBV-C RNA detection and clearance in infants. Peri-2 and Peri-3 infant specimens were not tested at other time-points for logistical reasons (longitudinal infant testing was conducted towards the end of our study and there was not time to ship additional specimens from Bangkok to Atlanta). Infants were not tested for GBV-C antibody which generally does not appear until the virus disappears; usually occurring after at least 10-12 months of age in three other studies [de Martino et al., 1998; Lin et al., 1998; Zanetti et al., 1998]. Infants of GBV-C RNA negative mothers were assumed to be GBV-C RNA negative. Second born twins were excluded from the analysis.

2.1.2 THE BANGKOK PERINATAL STUDIES: MAIN FINDINGS, INCLUSION AND EXCLUSION CRITERIA, LOSS TO FOLLOW-UP, AND SPECIMEN AVAILABILITY
2.1.2.1 The Bangkok collaborative perinatal HIV transmission study (Peri-1), 1992-1994 [Shaffer et al., 1999b]

The Bangkok collaborative perinatal HIV transmission study enrolled 342 HIV-positive and 344 HIV-negative pregnant women in a natural history study to identify risk factors for MTCT of HIV. No antiretroviral therapy (ART) was provided. No twins were born and only one woman breastfed her baby. HIV-positive women and their infants were followed for 15 months after delivery. Sixty-eight HIV-infected infants were followed until October 2004 when the study was terminated. HIV-negative women were interviewed and had blood drawn antenatally for comparison of baseline characteristics with HIV-positive women but neither they nor their infants were followed during or after delivery; therefore, no data or plasma were available from infants of HIV-negative mothers. The mother-to-child HIV transmission rate for infants of HIV-infected mothers in this cohort was 24.2%. In univariate analyses, higher transmission rates were associated with higher maternal HIV viral load, prematurity, low birth weight, vaginal delivery, and low NK cell percent. In multivariate analysis, the strongest predictor of transmission was high HIV viral load (highest quintile AOR 24.8) in addition to prematurity, vaginal delivery, and low NK cell percent [Shaffer et al., 1999b].

Consenting HIV-infected women attending antenatal care clinics (ANC) at the two study hospitals were eligible for the Peri-1 study if they resided in Bangkok and were willing to bring their child for study visits. The main reason for ineligibility was women planning to have their child cared for outside the Bangkok area after the delivery.
Controls were chosen randomly from those who tested HIV-negative at the same clinics. Of the 342 enrolled HIV-positive women, 46 were lost between enrollment and delivery (31 lost, 11 miscarriages, four abortions) [Shaffer et al., 1999b], and another two did not have a specimen available for our study. The GBV-C study therefore included and tested 294 (281 delivery and 13 third trimester) maternal specimens (Appendix 2 Peri-1 maternal population tree). There were no differences in baseline characteristics between women lost before final HIV transmission results and women who completed the original study [Shaffer et al., 1999b]. Serum specimens were available for 343 of 344 HIV-negative women; the missing specimen was previously sent to Atlanta for testing.

Six-month infant specimens were requested for infants of 107 HIV-positive women with GBV-C RNA in their plasma, including those with less than 1,000 viral copies detected per PCR test. If the six-month specimen was not available, the four-month specimen was requested; if the four-month specimen was not available, then the two- and twelve-month specimens were requested (see Section 2.3.2 infant GBV-C infection status). Peri-1 infants with GBV-C RNA in their plasma were also tested at all other available times to explore timing of viral detectability and clearance. (Peri-1 infant specimens were stored in the U.S. and were relatively easy to access). Of these 107, we obtained 93 infant specimens (87 six-month, three four-month and three with both two- and twelve-month) for GBV-C RNA RT-PCR testing (Appendix 2, Peri-1 infant population tree).
2.1.2.2 The Bangkok short-course zidovudine trial (Peri-2), 1996-1997 [Shaffer et al., 1999a]

The Bangkok short-course zidovudine trial, a triple-blinded randomized controlled trial, randomized 397 HIV-positive women to either zidovudine (AZT) (n=198) or placebo (n=199), with follow-up of mothers and infants to 18 months post-partum. Women in the zidovudine group received 300 mg oral zidovudine twice daily from 36 weeks gestation to onset of labour, once at onset of labour and then every three hours until delivery. Two second-born twins were excluded from analysis. The 55 infants found to be HIV-infected were enrolled in long-term follow-up to October 2004. 18.9% of women in the placebo arm transmitted HIV to their infants compared to 9.4% in the treatment arm. In univariate analyses, low HIV viral load and antiretroviral therapy were associated with reduced risk of perinatal HIV transmission. HIV viral load remained the only significant predictor of perinatal HIV transmission in multivariate analysis [Shaffer et al., 1999a]. No women breastfed their babies. The ‘Bangkok’ regimen was subsequently implemented nationally in Thailand and internationally.

Consenting HIV-1-infected women attending antenatal care clinics at the two study hospitals who consented were eligible for the study if they were: 18 years of age or older by delivery, 34 weeks or less gestation, lived in or near Bangkok, planned to deliver at the study hospitals, willing to bring the baby for follow-up and not breastfeed, willing and able to provide informed and voluntary consent, and healthy (hemoglobin>80gb/L, neutrophil count of \( \geq 1.0 \times 10^9/L \), platelet count of \( \geq 100 \times 10^9/L \), alanine aminotransferase
2.5 or less times the upper limit of normal, serum creatinine $\leq 133$ umol/L, and urine protein of 150mg/day or less by dipstick [$\leq 1+$]) [Shaffer et al., 1999a]. Women were ineligible if they had received ART, had had amniocentesis, or had fetal anomalies. Women were enrolled at 36 weeks. Plasma specimens were available for all 397 women (394 delivery and three 36 week specimens) (Appendix 2 Peri-2 maternal population tree). Six-month infant specimens were requested for 65 infants of women with GBV-C RNA in their plasma. Of these 65 infants, we obtained 61 infant specimens (57 six-month and four two-month). Of the four two-month specimens, one was GBV-C RNA-positive meeting our criteria of GBV-C RNA infection (see section 2.3.2 infant GBV-C infection status); the three negative specimens were categorized as missing as they could not be classified based on one negative test at 2 months (Appendix 2 Peri-2 infant population tree).

**2.1.2.3 The Bangkok Perinatal HIV Prevention Project (Peri-3), 1999-2004**

The Bangkok Perinatal HIV Prevention Project evaluated the implementation of perinatal HIV prevention programs at Siriraj and Rajavithi hospitals where the ‘Bangkok regimen’ (300 mg oral zidovudine twice daily from 34-36 weeks gestation to onset of labour, once at onset of labour and then every three hours until delivery) with the addition of four weeks neonatal AZT (2mg/kg q 6h x 4weeks) had been implemented. Women arriving for delivery with no antenatal care were given a rapid HIV test and offered AZT during delivery where possible. HIV-infected women were enrolled postpartum and all
infants of HIV-infected women received four weeks of AZT syrup. Approximately 65% of mothers received the full regimen, 17% a partial regimen, and 17% received no antiretroviral therapy. In addition, a substudy of 220 women receiving the full AZT regimen also received oral nevirapine 200 mg once during active labour and their infants received one dose (2mg/kg), once from 48 to 72 hours of life in addition to AZT. HIV-infected women and their infants were followed for four months. Nine second-born twins were excluded from the analysis. Interim analyses found that perinatal transmission was 4.6% in the NVP/AZT group [Chalermchokcharoenkit et al., 2004], 6.6% in the complete AZT regimen group, 10.7% in the intrapartum regimen group and 15.2% in the neonatal only group [Chotpitayasunondh et al., 2002]. Risk factors for transmission were high maternal HIV viral load at delivery, ruptured membranes greater than four hours, and use of oxytocin [Chotpitayasunondh et al., 2002]. Principal study findings included that barriers to receiving treatment were inconsistent antenatal care, fear of stigmatization and difficulty disclosing HIV status [Teeraratkul et al., 2005]. Fewer than 2% of women breastfed their infants one or more times. This study was still enrolling when we began our study and women and their infants enrolled after March 31, 2004 were not included in our study. As of March 31st, 2004, 53 HIV-infected infants had been identified.

HIV-positive women delivering at the two study hospitals were eligible for enrolment in Peri-3. As of March 31st, 2003, 759 women of the total 799 women eventually included in the study, had been enrolled, including 220 from the nevirapine sub-study. As all women were enrolled after delivery, there was no loss to follow-up prior to delivery and all had delivery blood specimens. Of these 759 women, 749
consented to have their blood stored for future research and were included in this sub-
study. We were able to determine GBV-C results for 713 women (Appendix 2, Peri-3 
maternal population tree). Four-month infant specimens were requested for infants of 
155 women with GBV-C RNA in their plasma. Of these 155, we obtained 149 infant 
specimens (135 four-month and 14 two-month specimens). Of the 14 two-month 
specimens, only one was positive and contributed a known GBV-C test result (see 
Section 2.3.2 infant GBV-C infection status). The others were negative and categorized 
as missing specimens as they could not be classified based on one negative test at 2 
months. In total there were 19 missing specimens (Appendix 2, Peri-3 infant population 
tree).

2.1.2.4 Total study population

Overall, 96% (1,440/1,498) of HIV-infected women and 99% (343/344) of HIV-
uninfected women enrolled in the original studies had specimens available for testing in 
the GBV-C study (Appendix 2, all study subjects study population tree). However, 40 
(12%) of 327 infant specimens requested were not available for testing. Of the 40 infants 
without specimens, two died before six months, 19 moved, withdrew or were lost, and 19 
did not have specimens available for testing. Eleven were HIV-infected, 14 HIV-
uninfected and 15 had HIV status unknown. The missing specimens accounted for 2.8% 
of the total 1,440 specimens requested for our study. Overall, 1,393 infants had a known 
GBV-C status; of these, 1,333 had a known HIV status.
2.2 LABORATORY METHODS

2.2.1 SPECIMEN HANDLING AND SHIPPING

Plasma and serum specimens were collected at the Bangkok study hospitals in heparinized and plain tubes respectively, kept in a cooler and transported to the TUC laboratory in Bangkok for processing. Aliquots for storage were frozen at -82°C within five hours of collection. Specimens were shipped to the laboratory at CDC, Atlanta on dry ice. Peri-1 1 ml plasma specimens from HIV-infected women and their infants were shipped over the study period 1992 to 1994, where they were stored in liquid nitrogen until pulled for this study. Maternal Peri-1 HIV-negative 1 ml and Peri-3 500 uL specimens were shipped in March 2004, maternal Peri-2 500 uL specimens were shipped in December 2004, and Peri-2 and 3 infant specimens in March 2005. All specimens arrived frozen and were stored at -80°C at the CDC laboratory. Peri-1 specimens were thawed, realiquotted into 250 uL aliquots and refrozen. A 250 uL aliquot from each woman was later thawed again and 100 uL or 200 uL of plasma or serum respectively put into a lysing buffer for RNA extraction; 15 uL was refrozen for ELISA testing, and the remainder refrozen for future testing. Peri-2 and 3 specimens were realiquotted on arrival
into a 200 uL aliquot for PCR testing, 15 uL for EIA (enzyme immunoassay) testing and the remainder for future testing and refrozen. We used a smaller plasma volume for infant PCR testing (100 uL). Additional Peri-1 infant specimens were retrieved from the Lawrenceville repository where they had been sent during the course of this study. Laboratory testing was performed from March 2004 to July 2005. All remaining specimens were returned to either Lawrenceville (Peri-1 specimens) or to the TUC laboratory in Bangkok (Peri-2 and 3 specimens) in July 2005.

Most specimens were tested following their second thaw; however, a few, including 50 specimens from Peri-3 and one from Peri-1, were tested on a third thaw specimen. However, there was not a significant difference in number of GBV-C viral copies detected between those specimens tested on a third thaw and those tested on a second thaw (Wilcoxon rank sum, p=0.61) among Peri-3 specimens. All Peri-1 EIA tests were also performed on third thaw specimens.

Plasma and serum specimens were coded with a unique specimen and study number and were analyzed blind to study variables including the main outcome (the HIV status of the infant), with the exception of maternal HIV status. As specimens were ‘machine read’, there was no opportunity for human bias in measurement of GBV-C viral load or EIA optical density. Decision rules for classifying specimens positive or negative were created blind to study variables.
2.2.2 TESTING

2.2.2.1 Laboratory techniques

Specimens were tested at the CDC laboratory in Atlanta, under the supervision of Dr. Sal Butera. This laboratory has had experience in GBV-C EIA, RT-PCR and genotype testing including the development of PCR and genotype tests for GBV-C. A site visit by Wendy Bhanich Supapol was made prior to commencement of testing to review testing protocols.

2.2.2.2 Detecting GBV-C antibody

The presence of GBV-C antibodies was detected with the Roche Diagnostics GmbH Microtitre plate assay (Roche Diagnostics Corp, Indianapolis, Ind.), an enzyme immunological test for research purposes only, for the qualitative determination of IgG antibodies to the GBV-C virus E2 envelope protein (anti-E2) in serum and plasma.\[Roche, \text{uPLATE Anti-HGenv}\] The EIA has been found to be a more sensitive method for the detection of anti-E2 antibodies than dot blot or Western blot assays [Lo et al., 2002]; however, the true sensitivity of this test is unknown [Hess and Diagnostics, 2004].

The GmbH uplate assay kit instruction protocol was followed using 5 uL of plasma or serum. An optical density cut-off was calculated for each plate according to
the manufacturer’s instructions. Optical densities greater than the cut-off were considered positive and optical densities less than the cut-off were considered negative. Results within 15% of the cut-off value were considered indeterminate (positive-indeterminate or negative indeterminate depending upon which side of the cut-off they lay).

Positive specimens (including positive-indeterminate specimen) and negative-indeterminate specimens were repeat tested with both the standard assay and a confirmatory assay on the same plate using freshly diluted material. The confirmatory assay indicates the amount of cross-reactivity between the test well and plasma, unrelated to the presence of GBV-C antibody. If both standard assays were positive and the ratio of the standard assay to the confirmatory assay on the same run was > 1.5 then the specimen was called positive; if the ratio was less than 1.5 then it was called negative. Twenty-seven (11%) of 241 positives were reclassified as negative in confirmatory testing. For negative-indeterminate specimens, if first and second round assays were both negative then the specimen was considered negative regardless of confirmatory test results. If first and second round testing conflicted and the specimen did not meet the ratio criteria, then the specimen was called negative. If first and second round testing conflicted and the specimen did meet the ratio criteria, then the specimen was retested in a third round and the result based on two of three results (four specimens were tested in a third round). Specimens that tested clearly negative (i.e. optical density (OD) more than
15% below the cut-point) were not routinely retested, with the exception of a random sample of 150 negatives, all of which retested as negative.

2.2.2.2.1 Quality Control

All test kits were from the same lot. As kit controls (both positive and negative) were specific to each kit, they could not be directly compared. The OD of negative controls ranged from 0.117 to 0.147 and the OD of positive controls ranged from 0.989 to 1.258 meeting the test protocol requirements. All kit controls performed as expected and no runs had to be repeated. Three results from each 84 well plate randomly selected from different row and columns were checked (including confirmatory testing) and all were correctly classified in the data base.

2.2.2.3 Quantitative RT-PCR assay for determination of GBV-C viral load

GBV-C viral RNA was extracted from 200 uL of plasma (100 uL for Peri-1 infant specimens) using the QIAamp MinElute Virus Vacuum kit (Qiagen Inc., Valencia, Calif.),[QIAGEN, 2002a] according to the manufacturer’s instructions. The volume of the final RNA eluate was 50 uL. Five microliters of RNA (10 uL if the starting volume of plasma was 100 uL for Peri-2 and Peri-3 infants) was amplified in a quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) assay based on TaqMan™ technology using the Quantitect Probe RT-PCR kit (Qiagen Inc., Valencia, Calif.),[QIAGEN, 2002b] according to the manufacturer’s instructions and using a single well. The reaction volume was 50 uL and the number of reaction cycles was 40. The
sequences of the primers used in our study to amplify a 105-base pair fragment of the conserved 5' untranslated region (UTR) of GBV-C and the dual-labeled probe are as follows (all nucleotide numbers below refer to the sequence with accession number U44402): [Linnen et al., 1996]

Forward primer: GBV-C 03.1-F - 5' GCA CGG TCC ACA GGT GTT 3' (18-mer encompassing nucleotides 226-243);

Reverse primer: GBV-C 03.2-R - 5' GTA CGT GGG CGT CGT TTG 3' (18-mer encompassing nucleotides 313-330);

Probe: G-THAI-UTR-P-3 - 5' CCG ACG TCA GGC TCG TCG TTA AAC 3' (24-mer encompassing nucleotides 268-291).

Viral copy numbers from each specimen were estimated with reference to a standard curve. The standard curve is a line produced by plotting the number of cycles it takes for a fluorescent signal to be received from each of eight standards against the known quantity of virus in each standard \(10^{1-8}\). The copy number per ml was then calculated by multiplying the estimated reaction copy number by an adjustment factor (50 for maternal and infant Peri-2 and Peri-3 specimens, and Peri-1 maternal specimens, where 1/50 ml of plasma was in the amplification process; and 100 for Peri-1 infant specimens where 1/100 ml of plasma was used in the amplification process) to convert it to the amount of virus that would have been in 1 mL of plasma. The lower limit of reliable detection was 1,000 copies per reaction (20 uL of plasma). Specimens with a reaction copy number of 1,000 or greater were considered GBV-C RNA positive. Specimens with
no virus detected after 40 cycles were considered GBV-C RNA negative. Specimens with a reaction copy number of one to 999 were considered GBV-C RNA indeterminate. Genotyping helped to resolve some of the GBV-C RNA indeterminate specimens (see Section 2.3.2 maternal GBV-C infection status below).

2.2.2.3.1 Quality control

Quality control for the RT-PCR was assured by several means. Firstly, the standard curve described above which validated the amplification part of the test should have formed a straight line with a perfect fit ($r = 1$). In all runs, $r$ ranged from 0.96 to 1.0. In addition, six human plasma standards of known quantities of virus ranging from ($10^2$-$10^6$) and a negative control were extracted and amplified in each run. These human standards validated the extraction as well as the amplification procedures. It was expected that all standards of $10^3$ and greater would amplify on each run (the $10^2$ standard amplifying approximately 50% of the time). In Peri-1, three of nine plates were repeated when the standards did not amplify as expected, but in Peri-2 & 3 no plates were repeated. All standards but those of one run (with standards in the lower range but deemed acceptable) tested within 0.5 log$_{10}$ viral load of each other; 0.5 log$_{10}$ is considered an acceptable variation in viral load testing. Two negative water controls were also included in each run to test for contamination; no viral copies should be detected in these controls. One Peri-1 pediatric plate was repeated due to contamination.

Because the PCR test for GBV-C is experimental and no commercially validated kit exists for this test, the CDC laboratory developed its own primers to amplify and
detect a segment of the GBV-C genome. We exchanged 250 specimens with the University of Iowa (see Appendix 3) to help validate this assay. Although there is no true gold standard for this test, the results of this validation study demonstrate an overall agreement of 78% (kappa score 0.56) and assuming Iowa was the gold standard, a sensitivity of 69% and a specificity of 87%. As Iowa also had misclassification problems (3 of 10 standards were misclassified by Iowa), true sensitivity and specificity are likely higher than this.

2.2.2.4 Determining GBV-C Genotype

Genotyping of GBV-C RNA-positive specimens was performed by reverse transcription-polymerase chain reaction (RT-PCR) with genotype-specific primers and restriction fragment length polymorphism (RFLP) analysis. GBV-C RNA-positive specimens were amplified by nested RT-PCR with genotype-specific primers, as described by Naito and Abe [Naito and Abe, 2001] and then by two second-round PCR reactions, one for genotypes 1 and 3 and the other for genotypes 2 and 4. The amplified products were resolved by polyacrylamide gel electrophoresis (PAGE) on 20% TBE gels (Invitrogen Corp., Carlsbad, Calif.) and assigned to genotypes 1 through 4. The length of the amplicons generated using the genotype-specific primers were as follows: genotype 1 - 95 base pairs (bp); genotype 2 – 174 bp; genotype 3: 161 bp; genotype 4 – 161 bp. Since the differences in amplicon size for the different genotypes were minimal, the laboratory at CDC confirmed genotypes by restriction fragment length polymorphism (RFLP) based on the protocol described by Schleicher and Flehmig [Schleicher and
Flehmig, 2003], with a minor modification. The amplicons were column-purified using the QIAquick PCR purification kit (Qiagen, Inc., Valencia, Calif.) and subjected to restriction digestion with the restriction enzymes Hinfl, HaeII and ScrFI (New England Biolabs, Beverly, Mass.) according to the manufacturer’s instructions. The digestion products were resolved by polyacrylamide gel electrophoresis (PAGE) on 20% TBE gels (Invitrogen Corp., Carlsbad, Calif.) to facilitate confirmation of the genotypes. Two subgenotypes (2a or 2b) were similarly resolved.

2.3 GBV-C INFECTION STATUS

Because the lower limit of viral detection for the GBV-C real time RT-PCR test is 1,000 copies, any specimen measuring 1,000 viral copies or more was considered GBV-C RNA-positive. Because we also tested maternal specimens for GBV-C genotype, if the maternal GBV-C RT-PCR test measured less than 1,000 viral copies, we used the genotype test (a second test for presence of RNA) to resolve whether the maternal specimen should be classified as RNA-positive or RNA-indeterminate. We also classified the <1% of specimens that tested positive for both GBV-C RNA and antibody as RNA-positive, because they still had active infection (Figure 1). Infant samples were tested for RNA only (Figure 2).
Figure 1: Determination of maternal GBV-C status

<table>
<thead>
<tr>
<th>Determination of Maternal GBV-C Status</th>
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<tbody>
<tr>
<td>GBV-C RT-PCR maternal viral load testing</td>
</tr>
<tr>
<td>VI = 0</td>
</tr>
<tr>
<td>VI = 1 - 999</td>
</tr>
<tr>
<td>VI ≥ 1,000</td>
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<tr>
<td>Genotype Testing</td>
</tr>
<tr>
<td>genotype not detected</td>
</tr>
<tr>
<td>genotype detected</td>
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<tr>
<td>GBV-C RNA positive</td>
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<tr>
<td>GBV-C RNA indeterminate</td>
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<tr>
<td>GBV-C RNA positive</td>
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<tr>
<td>GBV-C antibody testing</td>
</tr>
<tr>
<td>Anti-E2 +</td>
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<tr>
<td>Anti-E2 -</td>
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<tr>
<td>Anti-E2 +</td>
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<tr>
<td>Anti-E2 -</td>
</tr>
<tr>
<td>GBV-C antibody positive</td>
</tr>
<tr>
<td>GBV-C positive</td>
</tr>
<tr>
<td>GBV-C unknown*</td>
</tr>
<tr>
<td>GBV-C RNA positive</td>
</tr>
</tbody>
</table>

*GBV-C unknowns excluded from analysis
Figure 2: Determination of infant GBV-C status

Determination of infant GBV-C status

GBV-C RT-PCR viral load testing

vl = 0  vl = 1 - 999  vl ≥ 1,000

GBV-C RNA negative  GBV-C RNA indeterminate  GBV-C RNA positive
2.3.1 MATERNAL GBV-C INFECTION STATUS

We classified women into four mutually exclusive categories:

1. GBV-C RNA positive: ≥ 1,000 viral copies detected in the real time RT-PCR reaction, or < 1,000 copies but able to detect genotype;

2. GBV-C antibody positive: 0 copies of virus detected and anti-E2 serologic positive;

3. GBV-C negative: 0 copies of virus detected and anti-E2 serologic negative;

4. GBV-C RNA indeterminate: 0-999 copies of virus detected and unable to detect a genotype.

2.3.2 INFANT GBV-C INFECTION STATUS

We classified infants into three all inclusive mutually exclusive categories:

1. GBV-C RNA positive: ≥ 1,000 copies of virus detected by 12 months

2. GBV-C RNA negative: 0 copies of virus detected in the 4- (Peri-3) or 6-month (Peri-1 and 2) infant specimen, or 0 copies of virus found in both the 2- and 12-month infant plasma specimens, or infant of mother with 0 viral copies;

3. GBV-C RNA indeterminate: from 0 to 999 copies of virus detected at any age or more than once and no specimen with viral copy numbers ≥ 1,000.
2.4 HIV DETECTION AND OTHER LABORATORY MEASURES

HIV testing methods were similar but not identical over the three studies. Infant qualitative DNA PCR was performed in house at the CDC in Atlanta as previously described [Shaffer et al., 1999b] for Peri-1, with the modified Roche Amplicor HIV-1 qualitative assay version 1 (Roche Diagnostic Systems) [Shaffer et al., 1999a] for Peri-2 and with the Roche Amplicor HIV-1 qualitative assay versions 1 and 1.5 for Peri-3. In Peri-1, maternal specimens were measured for HIV-1 RNA using the Roche Amplicor HIV-1 MONITOR Test, with a modification that added a new set of gag primers (SK 145 SK 151) to ensure sufficient quantification for subtype E [Shaffer et al., 1999b] (making it similar to version 1.5). In Peri-2 and -3, maternal and infant plasma was measured for HIV RNA with the Roche Amplicor HIV-1 MONITOR Test version 1.5. These assays had a threshold of sensitivity of 400 HIV RNA copies/ml and a linear quantitation range of 400-750,000 copies/ml. Other laboratory measures such as CD4+ count and NK cell percent were as previously described [Shaffer et al., 1999a; Shaffer et al., 1999b].
2.5 DEFINITION OF HIV INFECTION IN INFANTS

We used HIV infection status as determined by the original study authors [Chalermchokcharoenkit et al., 2004; Shaffer et al., 1999a; Shaffer et al., 1999b; Simonds, 1999]. The three original perinatal studies used slightly different criteria to diagnose HIV infection in infants:

Peri-1 [Shaffer et al., 1999b]:
- Infected: two positive PCR, or one positive PCR and an AIDS-defining condition
- Uninfected: two negative PCR including one at 6 months or older or seroreverted to HIV-negative on EIA test
- Indeterminate: infants not meeting above criteria

Peri-2 [Shaffer et al., 1999a]
- Infected: any positive PCR
- Uninfected: one negative PCR at 2 months or older
- Indeterminate: no PCR result, or PCR negative at birth but lost to follow-up before 2-month PCR test

Peri-3 [Simonds, 1999]
- Infected: \( \geq 2 \) positive RNA or DNA PCR from different blood specimens
- Uninfected: \( \geq 2 \) negative RNA or DNA PCR from different blood specimens, at least one of which was at 2 months or older and no positive RNA or DNA PCR
- Indeterminate: all other children (i.e. lost to follow-up, conflicting results)

Current guidelines [The Working Group on Antiretroviral Therapy and Medical Management of HIV Infected Children, 2005] for laboratory diagnosis of HIV-infection (2005) include:

- Infected: two positive HIV virologic tests performed on separate blood specimens, regardless of age
• Uninfected: two or more negative virologic tests one of which was performed at $\geq 4$ months, or, two or more negative antibody tests one performed at age $\geq 6$ months and no clinical or virologic evidence of infection.

The different diagnostic criteria in the three Bangkok perinatal studies had no effect on the ultimate determination of infant HIV outcomes. Even though Peri-2 HIV diagnostic criteria appear to be less stringent than those used in Peri-1 and Peri-3, because HIV-infected infants all had more than one HIV-positive blood specimen (even though it might not have been used to make an initial diagnosis) all infants would have met current working group guidelines. If we use current working group guidelines as a common standard against which we compare infant HIV diagnostic criteria across studies, all infant HIV status determinations would have satisfied the guidelines with the exception of HIV-negative infants from Peri-2 and -3 who were lost to follow-up between two and four months. However, given that a TUC laboratory study of early detection methods for infant HIV conducted during Peri-2 found that specificity of PCR testing was 100% at any time and sensitivity was 100% by 2 months of age [Young et al., 2000], it is reasonable to assume that all infants in the perinatal studies were in fact correctly classified on HIV status.

### 2.6 ART REGIMEN

Overall, there were seven different ART regimens in our study: no ART; maternal zidovudine (AZT) antenatally and at delivery; maternal AZT antenatally and at
delivery plus infant AZT; the preceding with the addition of single dose neviripine to both mothers and infants; infant AZT only; maternal antenatal AZT and infant AZT; and maternal delivery AZT and infant AZT. Because several of these ART groups had small numbers and similar rates of MTCT of HIV, they were categorized into 4 groups for initial analysis: no ART (MTCT of HIV 22%, 105/474); infant only AZT (MTCT of HIV 15%, 18/119); maternal AZT, including some with both maternal and infant AZT (MTCT of HIV 7.7%, 43/561); and maternal and infant AZT and NVP (MTCT of HIV 4.8%, 10/210).

### 2.7 ETHICAL CONSIDERATIONS

This retrospective GBV-C study was approved by the Research Ethics Board, University of Toronto, Canada; the Institutional Review Board, CDC, Atlanta, U.S.; and the Ethical Review Committees for Research in Human Subjects at the Thailand Ministry of Public Health and Siriraj Hospital, Bangkok, Thailand. The original studies received ethical approval from the Institutional Review Board, CDC, Atlanta, U.S. and the Ethical Review Committees for Research in Human Subjects at the Thailand Ministry of Public Health and Siriraj Hospital, Bangkok, Thailand. During the course of the present study, all three perinatal studies were terminated and data was delinked. This meant that our data no long had to be considered as from human subjects for ethical approval purposes.
The GBV-C study posed no risk and offered no benefit to the participants of the original perinatal studies as no contact with the women or their infants was required. As infection with GBV-C is not of current clinical significance and the tests used for this study are licensed for research purposes only, there was no reason to notify women of their test results. As the studies are now de-linked, it would not have been possible to share test results with the women or their physicians even if we had wanted to.

Codes linking participants to their study ID numbers were kept in a locked file cabinet at the TUC office prior to their destruction on study termination in October 2004. The data bases we received contained no personal identifiers.

2.8 DATA CLEANING AND MANAGEMENT

Ten percent of all GBV-C laboratory data were verified for accuracy by comparing the plate position ID number and the machine result print out, with the result and idnum in the final data set. Two errors were found and corrected. In addition, the original perinatal data sets were examined for outliers and untenable data using SAS (SAS Institute Inc., Cary, NC). Unusual findings in Peri-3 such as newborn gestational age of >50 weeks were removed (these were most likely codes for missing or unknown ‘66’ ‘77’ and ‘99’) and negative labour hours were corrected (labour hours was redefined
as the time from onset of labour to delivery rather than time of entry to the delivery room to onset of labour). The data sets were merged by subject ID number and/or specimen number in SAS; duplicates were identified and removed.

2.9 STATISTICAL ANALYSIS

The statistical methods presented in each manuscript are not repeated here; however, additional information not included in the manuscripts as well as differences in the analysis strategies between the objectives is presented. All study objectives involved binary outcomes (MTCT of HIV, MTCT of GBV-C, and maternal GBV-C infection and clearance) and logistic regression was employed for multivariate analyses.

The primary objective, to determine whether maternal or infant GBV-C infection was associated with MTCT of HIV, tested an etiologic hypothesis and other variables were included to determine if they confounded the associations of interest. For maternal GBV-C infection, we examined whether presence of GBV-C RNA (current infection) or presence of antibody (past GBV-C infection) reduced the risk of MTCT of HIV. For infant GBV-C infection, we examined whether presence of GBV-C RNA (current infection) reduced MTCT of HIV. (Infant samples were not tested for antibody, as antibody present during infancy would reflect passively acquired maternal antibody).
We could not examine MTCT of GBV-C by maternal HIV status because we did not have infant data for HIV-uninfected women. GBV-C RNA-positive women were further stratified for analysis by GBV-C genotype and GBV-C viral load. GBV-C RNA infected infants could not be further stratified by other variables due to the small number of HIV-infected infants in this group. Biologically plausible potential modifiers of the effect of maternal GBV-C infection (ART, HIV viral load, CD4+ count) were examined by stratification and logistic regression. Stratum-specific results are presented where there appeared to be a biologically significant difference and were assessed as significant if the interaction term was significant. Potential modifiers of the effect of infant GBV-C infection could not be examined due to a small cell.

Distributions of continuous variables were assessed for normality using distributional plots. HIV and GBV-C viral load were transformed to their log_{10} value to normalize their distributions and other variables were normalized by removing outliers (none of which were found to be errors). Observations containing outliers, were put back into the analysis if that variable was not included in the final analysis, in order to increase the number of observations available for regression. Associations between continuous variables were evaluated using a Spearman’s correlation analysis. Associations between continuous predictor variables and binary outcomes were assessed with the Mann Whitney U test using SAS PROC NPAR1WAY. Exact confidence limits were obtained for odds ratios with 0 cells using STATXACT. Continuous variables were grouped into
six to eight categories to ascertain the nature of the relationship with the outcome. If
the observed relationship was not linear, continuous variables were modeled as
categorical variables with cut-points determined by either what has typically been used in
other studies, or by the observed nature of the relationship. Heterogeneity of associations
between studies were examined using the Breslow-Day test of homogeneity in SAS.
Specific ART regimens could not be examined for homogeneity of effect as they were
study specific; however, a binary variable representing maternal ART vs. no maternal
ART demonstrated a homogeneous effect across Peri-2 and Peri-3 (Peri-1 had no
maternal ART) and was used for multivariate analysis. In addition, unmeasured
confounders may create heterogeneity of baseline risk across studies. Indicator variables
for the perinatal studies were included in the logistic regression model to adjust for
potential unmeasured confounders that may have resulted in heterogeneity in baseline
risk among studies. This is a form of conditional logistic regression and is similar to
adjustment for other confounders. Second born twins were not included in the analysis
because the outcomes are not independent.

Confounding of the association between maternal or infant GBV-C infection and
MTCT of HIV was assessed by examining the change in the beta value of the regressions
coefficients for maternal or infant GBV-C infection. Variables were added to the model
one by one in order of their significance in univariate analysis based on their p value and
then together in groups of two and three variables. Variables not significantly associated
with MTCT of HIV that may have confounded the effect of maternal or infant GBV-C on
MTCT of HIV would have been kept in the final model; however, ultimately no such confounding variables were identified. Regression diagnostic plots were examined for influential observations; all were found to be correct and none were removed. Goodness-of-fit was assessed with the likelihood ratio test and the Hosmer-Lemeshow goodness-of-fit test.

As the distribution of infant GBV-C infection among HIV-infected infants resulted in a small cell, overdispersion was assessed. Overdispersion occurs when the observed variance is higher than the variance of a theoretical model; underdispersion occurs when the variance is lower. For a correctly specified model, the pearson chi-square statistic and the deviance, divided by their degrees of freedom (df) should be approximately equal to one. If values differ much from one, the assumption of binomial variability may not hold. A dispersion parameter is introduced to the model to lower this overdispersion effect. Adding these dispersion parameters did not substantially change any model estimates.

Objectives 2 and 3 were more exploratory as we wanted to determine possible correlates of MTCT of GBV-C and GBV-C infection and clearance. For Objective 2, to determine correlates of infant GBV-C infection, the outcome, infant GBV-C infection was determined through testing for GBV-C RNA (section. 2.3.2 Infant GBV-C status). Antibody testing was not included in the determination of infant GBV-C
status as it would have reflected passively acquired maternal antibody during this period. For Objective 3, examining determinants of GBV-C infection in women, we examined many behavioural and sociodemographic correlates of GBV-C infection. In this analysis, it does not matter if the woman’s GBV-C infection is current or past and therefore we used ‘ever GBV-C infected’ as our outcome (i.e. presence of GBV-C RNA or antibody). We also examined GBV-C clearance (i.e. presence of GBV-C antibody among those with evidence of GBV-C infection). Univariate and bivariate analyses were performed as above. No particular assessment of confounding was performed and all biologically plausible interactions between any variables were assessed. As part of our analysis of correlates of MTCT of GBV-C, we assessed an interaction between caesarean section and maternal GBV-C viral load. As part of our analysis of correlates of GBV-C infection we assessed interactions between all variables and HIV status. Manual model building by strength of univariate association was performed and was confirmed with automated forward and backward model building. Model fit was assessed as for Objective 1.
2.10 REFERENCES:


Roche. uPLATE Anti-HGenv. package insert.


Chapter 3

Paper 1: Reduced Mother-to-Child Transmission of HIV Associated with Infant but not Maternal GB Virus C Infection

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Reduced Mother-to-Child Transmission of HIV Associated with Infant but not Maternal GB Virus C Infection

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(See the editorial commentary by Björkman and Widell, on pages 1358–60.)

Background. Prolonged coinfection with GB virus C (GBV-C) has been associated with improved survival in human immunodeficiency virus (HIV)–infected adults. We investigated whether maternal or infant GBV-C infection was associated with mother-to-child transmission (MTCT) of HIV-1 infection.

Methods. The study population included 1364 HIV-infected pregnant women enrolled in 3 studies of MTCT of HIV in Bangkok, Thailand (the studies were conducted from 1992–1994, 1996-1997, and 1999–2004, respectively). We tested plasma collected from pregnant women at delivery for GBV-C RNA, GBV-C antibody, and GBV-C viral genotype. If GBV-C RNA was detected in the maternal samples, the 4- or 6-month infant sample was tested for GBV-C RNA. The rates of MTCT of HIV among GBV-C–infected women and infants were compared with the rates among women and infants without GBV-C infection.

Results. The prevalence of GBV-C RNA in maternal samples was 19%. Of 245 women who were GBV-C RNA positive, 101 (41%) transmitted GBV-C to their infants. Of 101 infants who were GBV-C RNA positive, 2 (2%) were infected with HIV, compared with 162 (13%) of 1232 infants who were GBV-C RNA negative (odds ratio [OR] adjusted for study, 0.13 [95% confidence interval {CI}, 0.03–0.54]). This association remained after adjustment for maternal HIV viral load, receipt of antiretroviral prophylaxis, CD4+ count, and other covariates. MTCT of HIV was not associated with the presence of GBV-C RNA (adjusted OR [aOR], 0.94 [95% CI, 0.62–1.42]) or GBV-C antibody (aOR, 0.90 [95% CI, 0.54–1.50]) in maternal samples.

Conclusions. Reduced MTCT of HIV was significantly associated with infant acquisition of GBV-C but not with maternal GBV-C infection. The mechanism for this association remains unknown.

GB virus C (GBV-C), a flavivirus closely related to hepatitis C virus and not known to cause any disease, may have an inhibitory effect on HIV-1 replication. GBV-C viremia often persists for years, but it is eventually cleared in 50%–75% of people, at which time antibodies to the GBV-C E2 envelope usually appear [1]. Persistent coinfection with GBV-C virus was associated with improved survival rates among HIV-infected adults in 2 of 3 recently reported longitudinal cohort studies [2–4], with the effect depending on the time chosen for CD4+ cell count adjustment. Loss of GBV-C RNA in previously infected individuals was associated with worse survival rates, compared with those who were never in-

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The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the US Centers for Disease Control and Prevention.

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fected. Some investigators believe that GBV-C infection may improve survival rates over the long term [3, 5], due to several biologically plausible mechanisms including interference with HIV viral replication [6], increased production of chemokine ligands for the CC chemokine receptor 5 (CCR5) [7], downregulation of the CCR5 receptor [7, 8], and modulation of the T-helper cytokines [9]. However, other researchers suggest that the causal relationship may be reversed and that GBV-C infection status is, rather, a phenomenon secondary to HIV-progression [2, 4]. The nature of the interaction between GBV-C and HIV remains unclear.

If GBV-C inhibits HIV replication, we hypothesized that maternal or infant infection with GBV-C might also reduce mother-to-child-transmission (MTCT) of HIV. Worldwide, approximately 700,000 children are infected with HIV annually, largely through MTCT of HIV. Biologic mechanisms that inhibit HIV replication may be relevant to the prevention of this type of transmission. We conducted a study to test this hypothesis using data and stored blood collected during 3 large perinatal studies in Bangkok.

**METHODS**

**Study population and design.** The study population consisted of participants in 3 perinatal HIV transmission studies in which women did not breast-feed (hereafter, “Peri-1,” “Peri-2,” and “Peri-3”) conducted jointly by the Thailand Ministry of Public Health and the US Centers for Disease Control and Prevention (CDC) at 2 large Bangkok hospitals between 1992 and 2004 [10–12]. Peri-1 (1992–1994) was a natural history study conducted prior to the use of antiretroviral prophylaxis for the prevention of MTCT of HIV. Peri-2 (1996–98) was a randomized, placebo-controlled trial assessing the efficacy of short-course zidovudine prophylaxis. Women in the zidovudine group received 300 mg of zidovudine orally twice daily from 36 weeks gestation, once at onset of labor, and then every 3 h until delivery. Peri-3 (1999–2004) was an observational study to evaluate the implementation of the “Bangkok regimen” as described above, but with 4 weeks of zidovudine therapy added for neonates. Approximately 65% of mothers received both the antenatal and the delivery components of the regimen, 17% received a partial regimen, and 17% received no antiretroviral medications. All infants received zidovudine. A substudy population of 220 women who received the full regimen also received 200 mg of nevirapine orally once during labor, and their infants received 1 dose of nevirapine in addition to zidovudine.

HIV-infected women from these studies who had a blood specimen obtained at delivery that was available for testing as of March 31, 2004, and whose infant had a known HIV test result were included in the present study. Maternal plasma specimens collected at delivery were tested for GBV-C RNA, GBV-C antibody, and GBV-C viral genotype. Infants of GBV-C viremic mothers who had a 4- or 6-month infant plasma specimen—the time during which GBV-C RNA has been detectable in GBV-C-infected infants in other studies [13, 14]—were tested for GBV-C RNA. Infants of mothers who tested negative for GBV-C RNA were assumed to be GBV-C RNA negative.

This study was approved by the Research Ethics Board, University of Toronto, Canada; the Institutional Review Board, CDC, Atlanta; and the Ethical Review Committees for Research in Human Subjects at the Thailand Ministry of Public Health and Siriraj Hospital, Bangkok, Thailand.

**Specimen collection and transport.** In the original studies, specimens were collected and tested for HIV, HIV viral load, CD4+ count, and NK cell percentage, as described elsewhere [10, 11]. Frozen specimens were shipped on dry ice from Bangkok to Atlanta for GBV-C testing. Laboratory staff were blinded to subject data.

**GBV-C testing.** GBV-C viral RNA was extracted from 200 µL of plasma by use of the QIAamp MinElute Virus Vacuum kit (Qiagen) and quantified with the Quantitect Probe RT-PCR kit (Qiagen), in accordance with the manufacturer’s instructions and as described elsewhere [15]. The lower limit of reliable detection of this quantitative reverse transcription polymerase chain reaction (RT-PCR) test is 1000 viral copies per reaction. Samples positive for GBV-C viral RNA, including a small number with 1-999 viral copies, were confirmed by genotype analysis (see below). Plasma specimens were tested for antibody to the GBV-C E2 envelope protein (anti-E2) by a µPLATE Anti-HGenv microtiter assay (Roche Diagnostics). To determine genotype, samples positive for GBV-C RNA were amplified by nested RT-PCR using genotype-specific primers [16] and resolved by polyacrylamide gel electrophoresis on 20% Tris-borate-EDTA gels (Invitrogen). To distinguish genotypes with a similar amplicon molecular size, restriction fragment length polymorphism analysis was carried out [17].

**GBV-C infection status.** We classified women into 1 of the following 4 mutually exclusive categories: (1) GBV-C RNA positive (with or without antibody), defined as ≥1000 viral copies detected with RT-PCR reaction or 1–999 viral copies detected that we were able to genotype; (2) GBV-C antibody positive, defined as anti-E2 positive serology test result and GBV-C RNA negative; (3) GBV-C negative, defined as GBV-C RNA negative and anti-E2 negative serology test result; and (4) GBV-C RNA indeterminate, defined as 1–999 copies of virus detected that we were unable to genotype.

Infants were classified into 1 of the following 3 mutually exclusive categories: (1) GBV-C RNA positive, defined as ≥1000 viral copies detected; (2) GBV-C RNA negative, defined as no virus detected or infant of a mother who tested negative for GBV-C RNA; and (3) GBV-C RNA indeterminate, defined as 1–999 copies of virus detected. Subjects (both mothers and infants) who were classified as GBV-C RNA indeterminate were excluded from their respective univariate analyses. However, as
maternal GBV-C status was not included in the final model, mothers with indeterminate GBV-C status were not excluded from the multivariate analysis of infant HIV infection.

**Statistical methods.** The statistical significance of the associations between maternal and infant GBV-C infection and MTCT of HIV was assessed with 2-sided $P$ values by use of $\chi^2$ or Fisher exact tests. Odds ratios (ORs) adjusted for participation in the Peri-1, Peri-2, or Peri-3 study were calculated by use of the Cochran-Mantel-Haenszel procedure. Logistic regression was also used to calculate odds ratios adjusted for multiple covariates and 95% Wald confidence intervals (CIs). Homogeneity of the odds ratios across studies was assessed with the Breslow-Day test. The 3 studies were combined for the multivariate analysis, and a variable indicating the perinatal study was included to adjust for any unmeasured confounding between studies. Variables associated with MTCT of HIV in univariate analysis that had a $P$ value $.20$ were included in the initial multivariate analysis. Manual modeling strategies were employed to control for confounding. HIV and GBV-C viral load were transformed to their log$_{10}$ value if a linear relationship was not observed, continuous variables were categorized at generally used cut points or at levels defined by the relationship observed with MTCT of HIV. All analyses were performed with SAS (version 8.2; SAS Institute) or StatXact (version 6.0; Cytel Software).

**RESULTS**

Table 1 shows selected characteristics of the 1364 HIV-infected pregnant women included in our study. The median number of lifetime sexual partners was 2 (interquartile range [IQR], 1–2), 9% were commercial sex workers, 2% were injection drug users (IDUs), and 13% had partners who were IDUs. Over the time period covered by the 3 studies, the median maternal age increased from 22 to 26 years ($P < .001$), the proportion of women with an educational level higher than primary increased from 34% to 44% ($P = .01$), and the proportion of women with partners who were IDUs increased from 7% to 16% ($P < .001$).

The 1364 women were classified as follows: 262 women (19%) were GBV-C RNA positive, including 12 women who tested positive for both virus and antibody; 176 (13%) were GBV-C antibody positive; 877 (64%) were GBV-C negative; and 49 (4%) were GBV-C RNA indeterminate (figure 1). Overall, 438 (32%) women had evidence of current or past GBV-C infection, as indicated by presence of GBV-C RNA or GBV-C antibody, respectively. Of 245 infants with definitive GBV-C results born to mothers who were GBV-C RNA positive, 101 (41%) acquired GBV-C RNA from their mothers (table 1). Women enrolled in Peri-1 appeared to have a higher prevalence of GBV-C RNA positivity, despite lower GBV-C viral loads, and were less likely to transmit GBV-C to their infants than women enrolled in Peri-2 or Peri-3. Prevalence of GBV-C genotype 2A declined over the course of the studies, whereas genotypes 3 and 4 became more prevalent.

We observed a statistically significant association between infant GBV-C infection and reduced MTCT of HIV ($P < .001$) (table 2). In Peri-1, infant GBV-C RNA appeared highly protective against HIV acquisition; 0 of 21 infants with GBV-C RNA acquired HIV, compared with 62 (25%) of 248 infants not infected with GBV-C (OR, 0.00 [95% CI, 0.00–0.59]). A similar relationship was also observed in Peri-2, in which 1 (3%) of 29 infants infected with GBV-C acquired HIV, compared with 53 (15%) of 349 infants not infected with GBV-C (OR, 0.20 [95% CI, 0.03–1.50]), and in Peri-3, in which 1 (2%) of 51 infants infected with GBV-C acquired HIV, compared with 47 (7%) of 635 infants not infected with GBV-C (OR, 0.25 [95% CI, 0.03–1.85]). When data from all of the studies were combined, the association was highly significant, with a greater than 7-fold reduction in the odds of infant HIV acquisition (aOR adjusted for study, 0.13 [95% CI, 0.03–0.54]) among infants infected with GBV-C, compared with infants not infected with GBV-C.

We did not observe an association between the presence of GBV-C RNA in maternal samples and MTCT of HIV in any of the 3 studies or when the studies were combined (aOR adjusted for study, 0.94 [95% CI, 0.62–1.42]) (table 3). Among mothers who tested positive for GBV-C RNA, GBV-C genotype and maternal GBV-C viral load were also not associated with MTCT of HIV. The presence of maternal GBV-C antibody was found to be protective ($P = .04$) against MTCT of HIV only in Peri-1; this effect was not observed in Peri-2 or Peri-3, or when all studies were combined (aOR adjusted for study, 0.90 [95% CI, 0.54–1.50]). Among women who received antiretroviral therapy (ART), GBV-C RNA appeared somewhat more protective against MTCT of HIV (OR, 0.67 [95% CI, 0.28–1.61]), compared with women who did not receive ART (OR, 1.22 [95% CI, 0.77–1.92]), but not significantly so ($P = 0.24$, for interaction). Among coinfected women in our study, HIV and GBV-C viral loads were significantly inversely correlated (Spearman $\rho = –0.21$; $P < .001$). However, when stratified by receipt of ART, viral loads appeared to be correlated only among women who received ART (Spearman $\rho = –0.18$; $P = .05$) and not among women who did not (Spearman $\rho = –0.05$; $P = .56$).

In multivariate analysis (table 4), the odds ratio for the association between infant GBV-C and MTCT of HIV remained essentially unchanged after adjustment for other covariates (aOR, 0.14), including maternal receipt of ART and maternal HIV viral load at delivery. Maternal CD4+ count did not confound the association between infant GBV-C infection and MTCT of HIV and was not included in the final model. All analyses were initially adjusted for the perinatal study in which subjects originally participated, but, because receipt of ART was highly correlated with study, receipt of ART was substituted for the study variable in the final model, and this did not substantially change any odds ratio estimates. Although maternal HIV viral load is on the
Table 1. Clinical and demographic characteristics of 1364 HIV-infected women and their infants in Bangkok Thailand, according to the perinatal study in which they originally participated.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Original study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal characteristic</td>
<td></td>
</tr>
<tr>
<td>GBV-C status</td>
<td></td>
</tr>
<tr>
<td>RNA a</td>
<td>88/279 (22)</td>
</tr>
<tr>
<td>Antibody</td>
<td>31/279 (11)</td>
</tr>
<tr>
<td>Negative</td>
<td>149/279 (53)</td>
</tr>
<tr>
<td>Unknown</td>
<td>11/279 (4)</td>
</tr>
<tr>
<td>GBV-C viral loada</td>
<td></td>
</tr>
<tr>
<td>Log_{10} copies/mL</td>
<td>5.5 (4.1–6.5)</td>
</tr>
<tr>
<td>Participants evaluated, no.</td>
<td>88</td>
</tr>
<tr>
<td>GBV-C genotypea,b</td>
<td></td>
</tr>
<tr>
<td>2A</td>
<td>44/88 (50)</td>
</tr>
<tr>
<td>2B</td>
<td>0/88 (0)</td>
</tr>
<tr>
<td>3</td>
<td>26/88 (30)</td>
</tr>
<tr>
<td>4</td>
<td>8/88 (9)</td>
</tr>
<tr>
<td>Multiple</td>
<td>10/88 (11)</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
</tr>
<tr>
<td>Value</td>
<td>22 (20–26)</td>
</tr>
<tr>
<td>Participants evaluated, no.</td>
<td>279</td>
</tr>
<tr>
<td>Gravidity</td>
<td></td>
</tr>
<tr>
<td>Value</td>
<td>1 (1–2)</td>
</tr>
<tr>
<td>Participants evaluated, no.</td>
<td>279</td>
</tr>
<tr>
<td>Antiretroviral regimen</td>
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</tr>
<tr>
<td>None</td>
<td>279/279 (100)</td>
</tr>
<tr>
<td>Maternal ZDV</td>
<td>. . .</td>
</tr>
<tr>
<td>Maternal and infant ZDV</td>
<td>. . .</td>
</tr>
<tr>
<td>Infant ZDV</td>
<td>. . .</td>
</tr>
<tr>
<td>Maternal and infant ZDV + nevirapine</td>
<td>. . .</td>
</tr>
<tr>
<td>HIV viral load at delivery</td>
<td></td>
</tr>
<tr>
<td>Log_{10} copies/mL</td>
<td>4.3 (3.9–4.9)</td>
</tr>
<tr>
<td>Participants evaluated, no.</td>
<td>278</td>
</tr>
<tr>
<td>CD4(^+) count at delivery</td>
<td></td>
</tr>
<tr>
<td>Cells/mm(^3)</td>
<td>450 (330–570)</td>
</tr>
<tr>
<td>Participants evaluated, no.</td>
<td>275</td>
</tr>
<tr>
<td>Maternal NK cells, % at delivery</td>
<td></td>
</tr>
<tr>
<td>Value</td>
<td>11 (8–14)</td>
</tr>
<tr>
<td>Participants evaluated, no.</td>
<td>275</td>
</tr>
<tr>
<td>HIV subtypeb</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>260/273 (95)</td>
</tr>
<tr>
<td>B</td>
<td>10/273 (4)</td>
</tr>
<tr>
<td>E/B</td>
<td>3/273 (1)</td>
</tr>
<tr>
<td>BR/MN/C</td>
<td>. . .</td>
</tr>
<tr>
<td>Delivery characteristic</td>
<td></td>
</tr>
<tr>
<td>Cesarean delivery</td>
<td>33/279 (12)</td>
</tr>
<tr>
<td>Labor</td>
<td></td>
</tr>
<tr>
<td>Duration, h</td>
<td>7.0 (4.0–10.0)</td>
</tr>
<tr>
<td>Participants evaluated, no.</td>
<td>250</td>
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<tr>
<td>Membrane rupture</td>
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<tr>
<td>Duration, h</td>
<td>2.0 (1.0–4.0)</td>
</tr>
<tr>
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<tr>
<td>Infant characteristic</td>
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<tr>
<td>Perinatal HIV infectionc</td>
<td>68/279 (24)</td>
</tr>
<tr>
<td>Perinatal GBV-C infectiona,b</td>
<td>21/81 (26)</td>
</tr>
<tr>
<td>Birth at &lt;37 weeks</td>
<td>12/277 (4)</td>
</tr>
<tr>
<td>Birth weight &lt;2500 g</td>
<td>28/279 (10)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are proportion (%) of subjects or median (interquartile range), unless otherwise indicated. Peri-1, Peri-2, and Peri-3 were 3 perinatal HIV transmission studies that did not involve breast-feeding, conducted jointly by the Thailand Ministry of Public Health and the US Centers for Disease Control and Prevention at 2 large Bangkok hospitals. GBV-C, GB virus C; ZDV, zidovudine.

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* GBV-C RNA a mothers only.
* Subjects with nontypable virus excluded.
* For those with known results.
causal pathway between receipt of ART and MTCT of HIV, it was retained in the model and improved model fit; removing it increased the estimated effect of ART as expected, but did not change any other estimates. Other variables that significantly increased the risk of MTCT of HIV in multivariate modeling included high maternal HIV viral load at delivery, no maternal receipt of ART, percentage of maternal NK cells at delivery <5%, vaginal delivery, duration of membrane rupture greater than 4 h, and female sex of the infant. We observed no significant difference in median CD4$^+$ counts between infants infected with GBV-C but not infected with HIV and infants not infected with either GBV-C or HIV (at birth, 2170 and 2179, respectively [$P = .80$]; at 2 months, 2335 and 2346 [$P = .73$]; at 6 months, 2670 and 2360 [$P = .07$]).

DISCUSSION

In 3 separate studies of HIV-infected pregnant women in Thailand, we found that infants who acquired GBV-C from their mothers had lower rates of MTCT of HIV, compared with infants who were not infected with GBV-C. This association was statistically significant in both the univariate and multivariate analysis of the combined studies. Neither the presence of maternal GBV-C RNA nor the presence of GBV-C antibody affected the risk of MTCT of HIV.

Ours is the first study to observe an association between infant GBV-C infection and MTCT of HIV. One possible explanation for the observed association between infant GBV-C infection and lower rates of HIV acquisition could have been confounding. High maternal GBV-C viral load is associated with both increased transmission of GBV-C to the infant (in our study [data not shown] and others [18, 19]) and a lower level of plasma HIV RNA (in our study and others [20–22]), which is the main determinant of MTCT of HIV. Thus, infants who are infected with GBV-C could appear less likely to be HIV infected. However, because we adjusted for maternal HIV viral load in our multivariate analysis, the observed association is unlikely to be due to confounding by maternal HIV viral load.

Although we observed a statistical association between infant GBV-C infection and MTCT of HIV, we cannot conclude that the relationship is causal. We do not know the direction or mechanism for the observed effect. It is possible that infant HIV infection prevents infant GBV-C acquisition rather than the reverse, or that a third factor is responsible for the low observed rates of both HIV and GBV-C transmission. In adults, it is unlikely that either GBV-C or HIV prevents acquisition of the other virus, given the high coinfection rates observed [2–4] and the lack of an association between GBV-C viremia and HIV acquisition [23] as well as the similar rates of GBV-C acquisition and
clearance in HIV-infected and -uninfected women [24] observed in 2 recent studies. The same may not be true in the context of MTCT. Two additional studies have also noted a low prevalence of GBV-C infection among HIV-infected infants [25, 26]. While HIV-infected children appear to acquire GBV-C, as the prevalence of GBV-C infection was observed in one study to be higher in older HIV-infected children, compared with younger HIV-infected children [26], it is not known whether HIV-infected children acquire GBV-C at the same rate as HIV-uninfected children. No studies have examined whether GBV-C infection prevents HIV acquisition in infancy or childhood.

The precise temporal relationship between infant acquisition of HIV and infant GBV-C infection is unknown, but it appears that both HIV and GBV-C are acquired at similar times. In populations that do not receive ART and who do not breast-feed, it is thought that most transmission of HIV occurs late in pregnancy or during delivery [27]. HIV-1 can be detected by DNA PCR at birth in 25%–38% of HIV-infected infants and by 2 months in the remainder [28–30]. In our study, GBV-C RNA was detectable by PCR at birth in 3 (21%) of 14 infants in Peri-1 who tested positive for GBV-C RNA (data not shown). In several other small studies of vertical transmission of GBV-C, the virus was detectable by PCR in 10%–100% of infants at birth [14, 31, 32] and in all infants by 2–3 months of age [13, 14, 33], most likely indicating a similar timing of vertical transmission for both HIV and GBV-C. Cesarean delivery was associated with reduced rates of GBV-C vertical transmission in our study and 2 others [18, 19], which is also suggestive of transmission at delivery.

GBV-C–infected infants are most likely infected through vertical transmission. Most, but not all, studies have observed high GBV-C sequence homology in mother–infant pairs [14, 19, 34, 35] and no GBV-C RNA in plasma samples from infants born to RNA-negative mothers [13, 31, 34, 36]. In our study, none of the infants born to 38 GBV-C RNA–indeterminate mothers tested positive for GBV-C RNA (figure 1). Although it is theoretically possible that infants in our study acquired GBV-C from their mother or someone else after birth (GBV-C RNA has been isolated from saliva [37] but not from breast milk [38]), household transmission in infancy is thought to occur rarely, if at all [34, 39], and in any case is unlikely in our study, given the short interval from birth to sample collection.

### Table 2. Association of infant GB virus C (GBV-C) infection status with mother-to-child transmission of HIV in Bangkok, Thailand, according to the perinatal study in which subjects originally participated.

<table>
<thead>
<tr>
<th>Study, child GBV-C status</th>
<th>HIV-infected infants, proportion (%)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peri-1, no ART</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA+</td>
<td>0/21 (0)</td>
<td>0 (0–0.60)</td>
<td>.005a</td>
</tr>
<tr>
<td>Negative</td>
<td>62/248 (25)</td>
<td>1.0b</td>
<td></td>
</tr>
<tr>
<td>Peri-2, placebo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA+</td>
<td>0/7 (0)</td>
<td>0 (0–2.95)</td>
<td>.35b</td>
</tr>
<tr>
<td>Negative</td>
<td>37/187 (20)</td>
<td>1.0b</td>
<td></td>
</tr>
<tr>
<td>Peri-2, ZDV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA+</td>
<td>1/22 (5)</td>
<td>0.43 (0.35–4.45)</td>
<td>.70b</td>
</tr>
<tr>
<td>Negative</td>
<td>16/162 (10)</td>
<td>1.0b</td>
<td></td>
</tr>
<tr>
<td>Peri-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA+</td>
<td>1/51 (2)</td>
<td>0.25 (0.03–1.85)</td>
<td>.25b</td>
</tr>
<tr>
<td>Negative</td>
<td>47/635 (7)</td>
<td>1.0b</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA+</td>
<td>2/101 (2)</td>
<td>0.13 (0.03–0.54)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Negative</td>
<td>162/1232 (13)</td>
<td>...</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Peri-1, Peri-2, and Peri-3 were 3 perinatal HIV transmission studies that did not involve breast-feeding, conducted jointly by the Thailand Ministry of Public Health and the US Centers for Disease Control and Prevention at 2 large Bangkok hospitals between 1992 and 2004. ART, antiretroviral therapy; CI, confidence interval; OR, odds ratio; RNA+, RNA positive; ZDV, zidovudine.

a Fisher exact test.
b Reference.
c OR adjusted for study.

### Table 3. Association of maternal GB virus C (GBV-C) infection status with mother-to-child transmission of HIV in Bangkok, Thailand, according to the perinatal study in which subjects originally participated.

<table>
<thead>
<tr>
<th>Study, maternal GBV-C status</th>
<th>HIV-infected infants, proportion (%)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peri-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA+</td>
<td>21/88 (24)</td>
<td>0.83 (0.45–1.52)</td>
<td>.54</td>
</tr>
<tr>
<td>Antibody+</td>
<td>3/31 (10)</td>
<td>0.28 (0.08–0.98)</td>
<td>.036</td>
</tr>
<tr>
<td>Negative</td>
<td>41/149 (28)</td>
<td>1.0b</td>
<td></td>
</tr>
<tr>
<td>Peri-2, placebo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA+</td>
<td>7/24 (29)</td>
<td>2.00 (0.75–5.37)</td>
<td>.17</td>
</tr>
<tr>
<td>Antibody+</td>
<td>6/29 (21)</td>
<td>1.27 (0.47–3.46)</td>
<td>.64</td>
</tr>
<tr>
<td>Negative</td>
<td>24/141 (17)</td>
<td>1.0b</td>
<td></td>
</tr>
<tr>
<td>Peri-2, ZDV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA+</td>
<td>3/34 (9)</td>
<td>0.99 (0.26–3.73)</td>
<td>&gt;.99b</td>
</tr>
<tr>
<td>Antibody+</td>
<td>2/17 (12)</td>
<td>1.37 (0.29–6.70)</td>
<td>.66b</td>
</tr>
<tr>
<td>Negative</td>
<td>12/135 (9)</td>
<td>1.0b</td>
<td></td>
</tr>
<tr>
<td>Peri-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA+</td>
<td>7/116 (6)</td>
<td>0.79 (0.34–1.83)</td>
<td>.58</td>
</tr>
<tr>
<td>Antibody+</td>
<td>9/99 (9)</td>
<td>1.23 (0.57–2.65)</td>
<td>.60</td>
</tr>
<tr>
<td>Negative</td>
<td>34/452 (8)</td>
<td>1.0b</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA+</td>
<td>38/262 (15)</td>
<td>0.94 (0.62–1.42)</td>
<td>.77</td>
</tr>
<tr>
<td>Antibody+</td>
<td>20/176 (11)</td>
<td>0.90 (0.54–1.50)</td>
<td>.69</td>
</tr>
<tr>
<td>Negative</td>
<td>111/877 (13)</td>
<td>1.0b</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Peri-1, Peri-2, and Peri-3 were 3 perinatal HIV transmission studies that did not involve breast-feeding, conducted jointly by the Thailand Ministry of Public Health and the US Centers for Disease Control and Prevention at 2 large Bangkok hospitals between 1992 and 2004. Antibody+, antibody positive; CI, confidence interval; OR, odds ratio; RNA+, RNA positive; ZDV, zidovudine.

a Reference.
b Fisher exact test.
c OR adjusted for study.
Several plausible biologic mechanisms have been suggested for GBV-C interference with HIV replication. In cell cultures simultaneously coinfected with GBV-C and HIV, the replication of HIV was reduced by 49%, compared with cell cultures infected with HIV alone, whereas cell cultures that were coinfected with GBV-C prior to being infected with HIV showed a 99% reduction in viral replication 6 days after infection [6]. GBV-C infection in cell culture also reduced surface expression of the CCR5 receptor and induced production of chemokine ligands for the CCR5 receptor, including RANTES, MIP-1α, MIP-1β, and SDF-1, which appeared to decrease HIV replication, although such effects may not be reproducible in vivo [7].

There may also be mechanisms by which HIV interferes with GBV-C susceptibility or persistence and it has been hypothesized that the declining CD4+ count in HIV progression results in loss of GBV-C [2]. Rapidly progressing HIV is unlikely to be

### Table 4. Variables associated with mother-to-child transmission of HIV in 1364 mother-infant pairs, Bangkok, Thailand.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HIV-infected infants, proportion (%)</th>
<th>aOR adjusted for study only (95% CI)</th>
<th>P</th>
<th>aOR final multivariate model (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Child GBV-C status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA positive</td>
<td>2/101 (2)</td>
<td>0.13 (0.03–0.53)</td>
<td>&lt;.001</td>
<td>0.14 (0.03–0.58)</td>
<td>.007</td>
</tr>
<tr>
<td>Negative</td>
<td>162/1232 (13)</td>
<td>1.0a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal HIV viral load at delivery, log_{10} viral copies/mL</td>
<td></td>
<td></td>
<td></td>
<td>3.33 (2.52–4.41) per log_{10} increase</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>&gt;5.0</td>
<td>61/170 (36)</td>
<td>12.03 (7.00–20.67)</td>
<td>&lt;.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0–4.9</td>
<td>92/552 (17)</td>
<td>4.44 (2.74–7.19)</td>
<td>&lt;.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4.0</td>
<td>23/639 (4)</td>
<td>1.0a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal receipt of ART</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>53/771 (7)</td>
<td>0.41 (0.26–0.63)</td>
<td>&lt;.001</td>
<td>0.50 (0.34–0.75)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>No</td>
<td>123/593 (21)</td>
<td>1.0a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal CD4+ count at delivery, cells/mm³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;500</td>
<td>47/460 (10)</td>
<td>0.35 (0.20–0.63)</td>
<td>&lt;.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200–499</td>
<td>101/745 (14)</td>
<td>0.56 (0.35–0.92)</td>
<td>.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;200</td>
<td>27/153 (18)</td>
<td>1.0a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal NK cell percentage at delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>18/91 (20)</td>
<td>1.95 (1.12–3.39)</td>
<td>.02</td>
<td>2.50 (1.35–4.63)</td>
<td>.004</td>
</tr>
<tr>
<td>&gt;5</td>
<td>157/1267 (12)</td>
<td>1.0a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cesarean delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20/268 (7)</td>
<td>0.59 (0.36–0.97)</td>
<td>.03</td>
<td>0.54 (0.31–0.94)</td>
<td>.03</td>
</tr>
<tr>
<td>No</td>
<td>156/1096 (14)</td>
<td>1.0a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of ruptured membranes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;4 h</td>
<td>54/307 (18)</td>
<td>1.58 (1.10–2.25)</td>
<td>.01</td>
<td>1.75 (1.17–2.61)</td>
<td>.007</td>
</tr>
<tr>
<td>&lt;4 h</td>
<td>118/1034 (11)</td>
<td>1.0a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prematurity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;37 weeks</td>
<td>15/79 (19)</td>
<td>2.16 (1.17–4.01)</td>
<td>.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;37 weeks</td>
<td>159/1283 (12)</td>
<td>1.0a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>102/693 (15)</td>
<td>1.40 (1.01–1.94)</td>
<td>.04</td>
<td>1.50 (1.04–2.17)</td>
<td>.03</td>
</tr>
<tr>
<td>Male</td>
<td>174/671 (11)</td>
<td>1.0a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low birth weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2500 g</td>
<td>24/132 (18)</td>
<td>1.67 (1.02–2.72)</td>
<td>.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2500 g</td>
<td>152/1232 (12)</td>
<td>1.0a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal CD8+ percentage at delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;55%</td>
<td>113/1020 (11)</td>
<td>0.48 (0.34–0.68)</td>
<td>&lt;.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;55%</td>
<td>61/337 (18)</td>
<td>1.0a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** The number of infants evaluated in each category does not necessarily add up to 1364 because data were missing for some participants.

aOR, adjusted odds ratio; ART, antiretroviral therapy; CI, confidence interval; GBV-C, GB virus C.

a Reference.
responsible for the lack of coinfection observed in our studies, as there was no GBV-C coinfection among HIV-infected children in Peri-1, regardless of the rate of HIV progression (approximately 25% were rapid progressors, whereas 75% were slow or intermediate progressors [40]). A similar bias is also unlikely to have been introduced by the 13 missing infant specimens from infants born to GBV-C RNA–positive mothers, because even if 41% of the 6 HIV-infected and 7 HIV-uninfected infants had acquired GBV-C, our result would still be significant (OR, 0.25 [95% CI, 0.09–0.70]). In addition, such an effect would be unlikely to occur through CD4+ cell depletion, because although median CD4+ counts were lower in Peri-1 rapid progressors, compared with slow progressors, at 2 months of age (1510 and 2610 cells/mm3, respectively [40]), they were presumably sufficient to support GBV-C replication. Also, among HIV-uninfected infants in our study, we observed no difference in median CD4+ counts between GBV-C–infected and GBV-C–uninfected infants. Thus, in our study, there was no evidence that rapid HIV progression or the absolute number of CD4+ cells determined GBV-C susceptibility or persistence in early infancy.

It is possible that maternal GBV-C RNA has a weaker effect on MTCT of HIV than was detectable by our study. With a population of 262 women positive for GBV-C RNA among 1364 HIV-infected women (19%) and a 13% overall rate of MTCT of HIV, we had the power to detect a 65% reduction in MTCT of HIV (OR, 0.52) with presence of maternal GBV-C RNA. Since initiating this study, 2 other studies [41, 42] also failed to demonstrate an effect of maternal GBV-C infection on MTCT of HIV, although the latter study did observe a borderline beneficial effect in women after highly active ART became available. Two additional studies [36, 43] were too small to draw definitive conclusions. In our study, GBV-C RNA also appeared somewhat more protective against MTCT of HIV in women who received ART, compared with women who did not, although the difference was not significant.

ART appears to play an important role in GBV-C infection. In our study, HIV and GBV-C viral loads were inversely correlated only among women who received ART. A recent longitudinal study also observed that HIV replication decreased and GBV-C replication increased among coinfected individuals who received highly active ART [44].

The higher prevalence of GBV-C viremia observed in Peri-1 is noteworthy, especially given that lower viral loads would presumably have made detecting GBV-C more difficult. The reasons for the lower viral load observed in Peri-1 are unknown, but they may include length of storage of specimens, the absence of ART, or other changes in the population or epidemiology of GBV-C during the study period.

Our study had several limitations. Because all study subjects were Thai, the generalizability of our findings may be limited to certain populations, HIV subtypes, or GBV-C genotypes. The 262 women infected with GBV-C in our study had GBV-C genotypes 2, 3, or 4, which may differ in their interaction with HIV, compared with genotypes 1 and 5, which are found predominantly in Africa [45]. A limitation of GBV-C studies in general is the lack of a gold standard test for the detection of GBV-C RNA. As random measurement error tends to bias the odds ratio toward the null, measurement error might have masked a true association between maternal GBV-C infection and MTCT of HIV. In the unlikely event that measurement error was associated with the outcome (MTCT of HIV), it is possible our results could be biased in either direction. This is unlikely, however, given the strong association we observed with infant GBV-C infection. We did not test infants of GBV-C RNA–negative mothers, and, because the sensitivity of the GBV-C test is less than perfect, some women and their infants may have been incorrectly classified as GBV-C RNA negative. If the sensitivity of the GBV-C test was 80%, then it is possible that 66 women and 27 of their untested infants (assuming 41% MTCT of GBV-C) were misclassified as negative. However, even if 100 infants were misclassified as negative and 13 (13%) of these infants were HIV infected (the rate observed in our study), the observed association would still be significant (OR, 0.53 [95% CI, 0.30–0.93]). In addition, the observed association was also present when our analysis was restricted to infants born to GBV-C RNA–positive mothers, all of whom had samples tested for GBV-C RNA (OR, 0.08 [95% CI, 0.02–0.34]).

In conclusion, we observed no association between maternal GBV-C infection and MTCT of HIV, but we found a strong inverse association between infant GBV-C infection and MTCT of HIV. In the context of MTCT, the interaction between GBV-C and HIV appears to occur at or around the time of delivery and is independent of infant CD4+ count. Our results are robust and do not change with adjustment for potential sources of bias including confounding, measurement error, and missing specimens. Nevertheless, our findings need to be confirmed by other studies, and the underlying causal pathway and mechanisms need to be identified. GBV-C may, for example, influence the cytokine milieu in such a way as to reduce the risk of infant HIV acquisition. If infant GBV-C infection is ultimately found to prevent HIV acquisition, avenues should be explored for a potential therapeutic role of GBV-C or its associated mechanisms in the prevention of MTCT of HIV.

Acknowledgments

We thank Darci Hansen, Dr. Suvanna Asavapirayanont, Dr. Nirun Vanprapar, Dr. Kulkanya Chokephaisulkulit, Dr. Rutt Chuachowong, Dr. Achara Teeraratkul, Natapak Skunodom, Bongkoch Jetsawang, Kan- chanaya Neeyapun, Chantana Sukkase, Wanna Leelawiwat, Chonticha Kittinunvorakoon, Chalinthorn Sinhwhattana, Baranee Balmangkol, Naritada Chantharajwong, Aidarita Santiago, and Deanna Pong for their assistance with this project.
References

Chapter 4

Paper 2: Mother-to-child transmission of GB virus C in a cohort of women coinfectcd with GB virus C and HIV in Bangkok, Thailand


Mother-to-Child Transmission of GB Virus C in a Cohort of Women Coinfected with GB Virus C and HIV in Bangkok, Thailand

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Background. GB virus C (GBV-C) is an apathogenic virus that inhibits human immunodeficiency virus (HIV) replication in vitro. Mother-to-child transmission (MTCT) of GBV-C has been observed in multiple small studies. Our study examined the rate and correlates of MTCT of GBV-C in a large cohort of GBV-C–HIV-coinfected pregnant women in Thailand.

Methods. Maternal delivery plasma specimens from 245 GBV-C–HIV-infected women and specimens from their infants at 4 or 6 months of age were tested for GBV-C RNA. Associations with MTCT of GBV-C were examined using logistic regression.

Results. One hundred one (41%) of 245 infants acquired GBV-C infection. MTCT of GBV-C was independently associated with maternal antiretroviral therapy (adjusted odds ratio [AOR], 5.21 [95% confidence interval [CI], 2.12–12.81]), infant HIV infection (AOR, 0.05 [95% CI, 0.01–0.26]), maternal GBV-C load (>8.0 log10 copies/mL: AOR, 86.77 [95% CI, 15.27–481.70]; 7.0–7.9 log10 copies/mL: AOR, 45.62 [95% CI, 8.41–247.51]; 5.0–6.9 log10 copies/mL: AOR, 9.07 [95% CI, 1.85–44.33]; reference, <5 log10 viral copies/mL), and caesarean delivery (AOR, 0.26 [95% CI, 0.12–0.59]).

Conclusions. Associations with maternal GBV-C load and mode of delivery suggest transmission during pregnancy and delivery. Despite mode of delivery being a common risk factor for virus transmission, GBV-C and HIV were rarely cotransmitted. The mechanisms by which maternal receipt of antiretroviral therapy might increase MTCT of GBV-C are unknown.
Although GBV-C infection alone appears to have no clinical significance, MTCT of GBV-C may be important for several reasons. First, GBV-C infection has been associated with slower progression and prolonged survival in HIV-infected adults in some but not all studies [13–21] and has been observed to inhibit HIV replication in vitro [17]. It is therefore possible that GBV-C coinfection may also improve survival among HIV-infected infants. Second and potentially more importantly, MTCT of GBV-C was found to be associated with reduced MTCT of HIV in our larger related analysis [22], although the biologic mechanism for this association remains unknown.

Wide variations in rates of MTCT of GBV-C (range, 13%–100%) were reported in 19 published studies that we identified [1, 2, 4, 11, 12, 23–36]. A limitation of all of these studies is their sample size, varying from 1 to 34 GBV-C–infected women. Despite small sample sizes, 3 studies identified high maternal GBV-C load, and 2 of these studies found that vaginal or emergency caesarean delivery was a risk factor for MTCT of GBV-C [1, 2, 11]. To better delineate transmission rates and risk factors for transmission, we combined data from several studies of perinatal HIV transmission in Thailand, where 20% of HIV-infected pregnant women were coinfected with GBV-C [22]. Our cohort of 245 GBV-C–HIV-coinfected pregnant women and their infants is the largest cohort to examine correlates of MTCT of GBV-C to date.

METHODS

The source population for this study consisted of 1498 non-breast-feeding participants (enrolled before 31 March 2004) of 3 perinatal HIV transmission studies conducted jointly by the Thailand Ministry of Public Health and the US Centers for Disease Control and Prevention at 2 large Bangkok hospitals. Study 1 (1992–1994; n = 342) was a natural history study conducted before the advent of antiretroviral prophylaxis (ART) for the prevention of MTCT of HIV [37]. Study 2 (1996–1998; n = 397) was a randomized placebo-controlled trial assessing the efficacy of short-course zidovudine prophylaxis [38]. Study 3 (1999–2004; n = 759) was an observational study assessing the implementation of programs to prevent MTCT of HIV [39]. Eighty-three percent of women enrolled in study 3 received zidovudine prenatally and/or at delivery, and 220 women and their infants also received nevirapine. All infants in study 3 received either zidovudine treatment or combination treatment with zidovudine and nevirapine.

Delivery blood specimens from 1440 women were available and tested for GBV-C RNA, antibody, and if the specimen was GBV-C RNA positive, genotype. Plasma specimens from the GBV-C RNA–positive women’s infants at 4 or 6 months of age were tested for GBV-C RNA. For a few infants for whom 4- or 6-month specimens were not available, 2- and 12-month plasma specimens were requested. Plasma samples from 21 GBV-C RNA–positive infants from study 1 were also tested for GBV-C RNA at all other available collection times (from birth to 37 months) to assess the time of detection and clearance of GBV-C RNA in infants. Newborns had blood samples obtained directly and not from cord blood.

In the original 3 studies, specimens were collected and tested for the presence of HIV. HIV load and CD4+ and CD8+ cell counts were measured as described elsewhere [37, 38]. Laboratory staff were blinded to patient data.

GBV-C RNA was extracted from 200 µL of plasma with use of the QIAamp MinElute Virus Vacuum kit (Qiagen) and was quantified using the Quantitect Probe reverse-transcriptase polymerase chain reaction (RT-PCR) kit (Qiagen), according to the manufacturer’s instructions. The lower limit of reliable detection of this quantitative RT-PCR test is 1000 viral copies per reaction. Positive maternal samples, including 39 with 1–999 viral copies per reaction, were confirmed by genotype analysis, as described elsewhere [40, 41].

GBV-C RNA–positive women (defined as women with a GBV-C load ≥1000 viral copies per reaction or 1–999 viral copies per reaction and who had specimens could be genotyped) and their infants, were included in the present study. Infants were classified into 1 of 3 mutually exclusive categories: (1) GBV-C RNA positive (GBV-C load, ≥1000 viral copies per reaction; detected at any age), (2) GBV-C RNA negative (GBV-C load, 0 viral copies per reaction detected in the infant specimens from 4 months of age [study 3] or 6 months of age [studies 1 and 2] or 0 viral copies per reaction found in both the 2- and 12-month infant plasma specimens), and (3) GBV-C RNA indeterminate (GBV-C load, 0–999 viral copies per reaction detected at any age or at >1 time and no specimen with a GBV-C load ≥1000 viral copies per reaction).

Analyses were performed using SAS (version 8.2; SAS Institute). The statistical significance of associations of maternal and infant characteristics with MTCT of GBV-C within each study was assessed with 2-sided P values from χ2 or Fisher’s exact tests, using tests for trend for ordered categorical variables. Odds ratios (ORs) and 95% confidence intervals (CIs) adjusted for participants from study 1, 2, or 3 were calculated using the Cochran Mantel-Haenszel procedure for categorical variables. Homogeneity of ORs across studies was assessed with the Breslow-Day test, and the 3 studies were combined for the multivariable analysis, with a variable indicating the perinatal study included to adjust for any unmeasured confounding between studies. Variables associated with MTCT of GBV-C in the analysis adjusted for study that had a P value of <.20 were included in the analysis adjusted for study and covariates and were retained in the final model if their P value was <.05. HIV and GBV-C loads were transformed to their log10 values. If a linear relationship was not observed, continuous variables were categorized at generally used cutoff points or at levels defined by
Table 1. Characteristics of 245 women coinfected with GB virus V (GBV-C) and human immunodeficiency virus (HIV) and their infants, by original perinatal study, Bangkok, Thailand.

<table>
<thead>
<tr>
<th>Variable</th>
<th>1 (n = 81)</th>
<th>2 (n = 54)</th>
<th>3 (n = 110)</th>
<th>All (n = 245)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perinatal GBV-C transmission rate</td>
<td>21 (26)</td>
<td>29 (54)</td>
<td>51 (46)</td>
<td>101 (41)</td>
</tr>
<tr>
<td>Perinatal HIV transmission rate</td>
<td>16 (20)</td>
<td>10 (19)</td>
<td>5 (4.6)</td>
<td>31 (13)</td>
</tr>
<tr>
<td>ART regimen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>81 (100)</td>
<td>23 (43)</td>
<td>104 (42)</td>
<td></td>
</tr>
<tr>
<td>Maternal or maternal and infant zidovudine</td>
<td>...</td>
<td>31 (57)</td>
<td>53 (48)</td>
<td>84 (34)</td>
</tr>
<tr>
<td>infant zidovudine</td>
<td>...</td>
<td>...</td>
<td>24 (22)</td>
<td>24 (9.8)</td>
</tr>
<tr>
<td>Mother and infant zidovudine and nevirapine</td>
<td>...</td>
<td>...</td>
<td>33 (30)</td>
<td>33 (13)</td>
</tr>
<tr>
<td>Maternal characteristic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBV-C load, log₁₀ copies/mL</td>
<td>5.4 (4.1–6.7)</td>
<td>8.0 (7.5–8.5)</td>
<td>8.0 (6.9–8.3)</td>
<td>7.5 (5.9–8.1)</td>
</tr>
<tr>
<td>GBV-C genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2A</td>
<td>44 (54)</td>
<td>22 (41)</td>
<td>30 (29)</td>
<td>96 (40)</td>
</tr>
<tr>
<td>2B</td>
<td>0 (0)</td>
<td>1 (1.9)</td>
<td>3 (2.9)</td>
<td>4 (1.7)</td>
</tr>
<tr>
<td>3</td>
<td>22 (27)</td>
<td>18 (33)</td>
<td>46 (44)</td>
<td>86 (36)</td>
</tr>
<tr>
<td>4</td>
<td>7 (8.6)</td>
<td>11 (20)</td>
<td>21 (20)</td>
<td>39 (16)</td>
</tr>
<tr>
<td>Multiple</td>
<td>8 (9.8)</td>
<td>2 (3.7)</td>
<td>5 (4.8)</td>
<td>15 (6.3)</td>
</tr>
<tr>
<td>HIV load at delivery, log₁₀ copies/mL</td>
<td>4.3 (3.8–4.9)</td>
<td>4.2 (3.8–4.7)</td>
<td>4.0 (3.2–4.7)</td>
<td>4.2 (3.6–4.8)</td>
</tr>
<tr>
<td>CD4⁺ cell count at delivery, cells/mm³</td>
<td>450 (320–590)</td>
<td>363 (226–510)</td>
<td>359 (242–523)</td>
<td>390 (260–550)</td>
</tr>
<tr>
<td>Age, years</td>
<td>22 (20–25)</td>
<td>24 (22–29)</td>
<td>25 (22–28)</td>
<td>24 (21–27)</td>
</tr>
<tr>
<td>Gravidity</td>
<td>1 (1–2)</td>
<td>2 (1–2)</td>
<td>2 (1–3)</td>
<td>2 (1–2)</td>
</tr>
<tr>
<td>Education: primary or less</td>
<td>52 (64)</td>
<td>30 (56)</td>
<td>59 (54)</td>
<td>141 (58)</td>
</tr>
<tr>
<td>Commercial sex worker</td>
<td>10 (12.4)</td>
<td>7 (13)</td>
<td>10 (9.1)</td>
<td>27 (11)</td>
</tr>
<tr>
<td>IDU</td>
<td>1 (1.2)</td>
<td>0 (0)</td>
<td>7 (6.3)</td>
<td>8 (3.3)</td>
</tr>
<tr>
<td>Partner of IDU</td>
<td>5/76 (6.6)</td>
<td>6 (13)</td>
<td>17 (18)</td>
<td>28 (13)</td>
</tr>
<tr>
<td>Delivery characteristic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caesarean delivery</td>
<td>10 (12)</td>
<td>12 (22)</td>
<td>36 (33)</td>
<td>58 (24)</td>
</tr>
<tr>
<td>Duration of labor, h</td>
<td>6 (4–10)</td>
<td>9 (5–14)</td>
<td>7 (5–12)</td>
<td>7 (5–11)</td>
</tr>
<tr>
<td>Duration of membrane rupture, h</td>
<td>1.0 (1.0–4.0)</td>
<td>1.8 (0.4–6.2)</td>
<td>0.6 (0.05–3.0)</td>
<td>1 (0.2– 4.0)</td>
</tr>
<tr>
<td>Infant characteristic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prematurity (≤37 weeks)</td>
<td>5 (6.2)</td>
<td>0 (0)</td>
<td>9 (8.2)</td>
<td>14 (5.7)</td>
</tr>
<tr>
<td>Birth weight &lt;2500 g</td>
<td>9 (11)</td>
<td>1 (1.9)</td>
<td>15 (14)</td>
<td>25 (10)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%) of participants or median value (interquartile range). Study 1 was performed during 1992–1994, study 2 was performed during 1996–1997, and study 3 was performed during 1999–2004. ART, antiretroviral therapy; IDU, injection drug user.

the relationship observed with MTCT of GBV-C. Logistic regression was used to calculate ORs and 95% Wald CIs adjusted for multiple covariates. Biologically plausible interactions were assessed by adding an interaction term to the multivariable model.

This study was approved by the Research Ethics Board, University of Toronto, Canada; the Institutional Review Board, Centers for Disease Control and Prevention, Atlanta; and the Ethical Review Committees for Research in Human Subjects at the Thailand Ministry of Public Health and Siriraj Hospital, Bangkok, Thailand.

RESULTS

We were able to determine GBV-C infection status for 1387 (96%) of 1440 HIV-infected pregnant women for whom delivery blood specimens were available; of these women, 274 (20%) tested positive for GBV-C RNA. Of these women, 249 had infant specimens available for testing. One hundred one (41%) of 249 infants were classified as GBV-C RNA positive, 144 (58%) of 249 were classified as GBV-C RNA negative, and 4 (2%) of 249 were classified as GBV-C RNA indeterminate. GBV-C–HIV-coinfected women and their infants with known infant GBV-C status were included in the present analysis. The 4 GBV-C RNA–indeterminate infants, one of whom was HIV infected, were not included in further analysis.

The characteristics of these 245 GBV-C–HIV-coinfected women and their infants are summarized in table 1. The overall rate of MTCT of GBV-C was 41% (95% CI, 35%-48%) but was significantly different among studies (P = .002). The rate of MTCT of GBV-C was 26% in study 1, 54% in study 2, and
Table 2. Correlates of GB virus C (GBV-C) infection in 245 infants, Bangkok, Thailand.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Proportion (%) of GBV-C–infected infants (n = 245)</th>
<th>Adjusted for study only</th>
<th>Final multivariate model&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AOR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Maternal GBV-C load&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;8.0 log&lt;sub&gt;10&lt;/sub&gt; copies/mL</td>
<td>54/81 (67)</td>
<td>58.14 (4.42–765.25)</td>
<td>.001</td>
</tr>
<tr>
<td>7.0– 7.9 log&lt;sub&gt;10&lt;/sub&gt; copies/mL</td>
<td>31/65 (48)</td>
<td>33.15 (4.39–250.17)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>5.0– 6.9 log&lt;sub&gt;10&lt;/sub&gt; copies/mL</td>
<td>14/57 (25)</td>
<td>9.82 (1.92–50.16)</td>
<td>.002</td>
</tr>
<tr>
<td>&lt;5 log&lt;sub&gt;10&lt;/sub&gt; copies/mL</td>
<td>2/42 (4.8)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Infant HIV status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>2/31 (6.5)</td>
<td>0.08 (0.016–0.34)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Uninfected</td>
<td>99/214 (46)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Maternal HIV load&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0–5.9 log&lt;sub&gt;10&lt;/sub&gt; copies/mL</td>
<td>8/38 (21)</td>
<td>0.19 (0.06–0.66)</td>
<td>.007</td>
</tr>
<tr>
<td>4.0–4.9 log&lt;sub&gt;10&lt;/sub&gt; copies/mL</td>
<td>46/109 (42)</td>
<td>0.74 (0.32–1.71)</td>
<td>.48</td>
</tr>
<tr>
<td>3.0– 3.9 log&lt;sub&gt;10&lt;/sub&gt; copies/mL</td>
<td>30/66 (45)</td>
<td>0.63 (0.25–1.60)</td>
<td>.34</td>
</tr>
<tr>
<td>2.0–2.9 log&lt;sub&gt;10&lt;/sub&gt; copies/mL</td>
<td>17/32 (53)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Maternal ART</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>68/117 (58)</td>
<td>4.85 (2.18–10.75)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>No</td>
<td>33/128 (26)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Maternal CD4&lt;sup&gt;+&lt;/sup&gt; cell count at delivery&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;200</td>
<td>7/25 (28)</td>
<td>0.26 (0.14–0.92)</td>
<td>.03</td>
</tr>
<tr>
<td>&gt;=200</td>
<td>92/217 (45)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Caesarean delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16/58 (28)</td>
<td>0.35 (0.18–0.69)</td>
<td>.002</td>
</tr>
<tr>
<td>No</td>
<td>85/187 (46)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Duration of membrane rupture &gt;4 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>30/58 (52)</td>
<td>1.66 (0.90–3.06)</td>
<td>.10</td>
</tr>
<tr>
<td>No</td>
<td>69/183 (38)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Prematurity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2/14 (14)</td>
<td>0.21 (0.04–1.05)</td>
<td>.045</td>
</tr>
<tr>
<td>No</td>
<td>99/231 (43)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Sex of baby</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>45/128 (35)</td>
<td>0.62 (0.37–1.06)</td>
<td>.08</td>
</tr>
<tr>
<td>Male</td>
<td>56/117 (48)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Maternal CD8&lt;sup&gt;+&lt;/sup&gt; cell percentage at delivery&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;=40%</td>
<td>38/99 (38)</td>
<td>0.70 (0.41–1.21)</td>
<td>.20</td>
</tr>
<tr>
<td>&lt;40%</td>
<td>61/143 (43)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Maternal CD4/CD8 ratio&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;0.5</td>
<td>51/105 (49)</td>
<td>1.87 (1.09–3.22)</td>
<td>.02</td>
</tr>
<tr>
<td>&lt;0.5</td>
<td>48/134 (36)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Duration of labor, h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;=4</td>
<td>80/177 (45)</td>
<td>1.81 (0.88–3.71)</td>
<td>.09</td>
</tr>
<tr>
<td>&lt;4</td>
<td>12/42 (29)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Study 2</td>
<td>...</td>
<td>...</td>
<td>0.24 (0.07–0.84)</td>
</tr>
<tr>
<td>Study 3</td>
<td>...</td>
<td>...</td>
<td>0.12 (0.04–0.42)</td>
</tr>
</tbody>
</table>

NOTE. AOR, adjusted odds ratio; ART, antiretroviral therapy; CI, confidence interval.
<sup>a</sup> The final multivariate model included maternal GBV-C load, maternal receipt of ART, caesarean delivery, infant HIV status, and the original study in which the mother participated.
<sup>b</sup> Shown are trend AORs per log<sub>10</sub> increase. Cochran-Armitage test for trend, P < .001.
<sup>c</sup> Shown are trend AORs per log<sub>10</sub> increase. Cochran Armitage test for trend, P = .009.
<sup>d</sup> Data are for 242 infants.
<sup>e</sup> Data are for 239 infants.
46% in study 3. In study 1, we also observed significantly lower maternal GBV-C titers than in study 2 or 3 (P < .001).

The correlates of MTCT of GBV-C are presented in table 2. Mothers receiving ART were significantly more likely than mothers not receiving ART to transmit GBV-C to their infants (adjusted OR, 5.21 [95% CI, 2.12–12.81]; adjusted for study and covariates). The effect of maternal receipt of ART on MTCT of GBV-C was independent of GBV-C load, HIV load, and CD4+ cell count (adjusted OR 4.81 [95% CI, 1.97–11.72]). Table 3 presents rates of MTCT of GBV-C, MTCT of HIV, and maternal GBV-C and HIV load by ART regimen and study. When women did not receive ART, the rates of MTCT of GBV-C and HIV were very similar, whereas when women received ART, the rate of MTCT of HIV decreased (relative risk, 0.16; P < .001) and the rate of MTCT of GBV-C increased (relative risk, 2.25; P < .001); the ratio of MTCT of GBV-C to MTCT of HIV changed from 1.2 to 17.1. The rate of MTCT of GBV-C did not vary with the addition of nevirapine to zidovudine therapy (P = .36), and infant ART, compared with no ART, was not associated with increased MTCT of GBV-C (P = .54).

GBV-C and HIV were rarely cotransmitted, and only 2 of 245 infants acquired both viruses. The rate of MTCT of GBV-C was significantly lower among HIV-infected infants than among HIV-uninfected infants (6.5% [2 of 31 infants] vs. 46% [99 of 214]; adjusted for study and covariates). The effect of maternal receipt of ART on MTCT of GBV-C was independent of GBV-C load, HIV load, and covariates). The risk of MTCT of GBV-C was not significantly different between studies (adjusted OR for study 1 vs. study 2, 1.35 [95% CI, 0.56–3.27]; adjusted OR for study 1 vs. study 3, 0.68 [95% CI, 0.27–1.73]). However, after adjusting for maternal GBV-C load and ART, women in studies 2 and 3 had a significantly lower risk of transmitting GBV-C to their infants than did women in study 1 (adjusted OR for study 1 vs. study 2, 0.22 [95% CI, 0.07–0.65]; adjusted OR for study

**Table 3. Mother-to-child transmission (MTCT) of GB virus C (GBV-C) and human immunodeficiency virus (HIV) and maternal viral load, by antiretroviral therapy (ART) regimen and study, for 245 GBV-C–HIV-coinfected mothers, Bangkok, Thailand.**

<table>
<thead>
<tr>
<th>Study, ART regimen</th>
<th>Rate of MTCT of GBV-C, % (proportion)</th>
<th>Rate of MTCT of HIV, % (proportion)</th>
<th>Maternal GBV-C load at delivery, median copies/mL (IQR)</th>
<th>Maternal HIV load at delivery, median copies/mL (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: no ART</td>
<td>26 (21/81)</td>
<td>20 (16/81)</td>
<td>5.4 (4.1–6.7)</td>
<td>4.3 (3.8–4.9)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>30 (7/23)</td>
<td>30 (7/23)</td>
<td>7.9 (7.2–8.4)</td>
<td>4.7 (4.1–4.9)</td>
</tr>
<tr>
<td>Maternal AZT</td>
<td>71 (22/31)</td>
<td>10 (3/31)</td>
<td>8.1 (7.8–8.5)</td>
<td>4.1 (3.6–4.3)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant AZT</td>
<td>21 (5/24)</td>
<td>17 (4/24)</td>
<td>8.0 (6.9–8.2)</td>
<td>4.7 (4.2–5.1)</td>
</tr>
<tr>
<td>Maternal and infant AZT</td>
<td>55 (29/53)</td>
<td>2 (1/53)</td>
<td>8.0 (6.7–8.3)</td>
<td>3.9 (3.2–4.4)</td>
</tr>
<tr>
<td>Maternal and infant AZT and NVP</td>
<td>52 (17/33)</td>
<td>0 (0/33)</td>
<td>8.0 (7.2–8.4)</td>
<td>3.6 (2.3–4.3)</td>
</tr>
</tbody>
</table>

**NOTE.** AZT, zidovudine; IQR, interquartile range; NVP, nevirapine.
Figure 1. Time of GB virus C detectability and clearance in infants.

1 vs. study 3, 0.15 [95% CI, 0.05–0.45]). Adding and removing infant HIV acquisition and mode of delivery from the multivariate model did not affect the association between the variable indicating the original perinatal study and MTCT of GBV-C.

To examine the time of GBV-C detectability and clearance in infants, we tested plasma samples from all 21 GBV-C RNA–positive infants from study 1 for GBV-C RNA at all available times (figure 1). None of these infants were coinfected with HIV. Three (21%) of 14 newborn samples available for testing were positive for GBV-C RNA and 19 (95%) of 20 tested positive by 2 months. Four (50%) of 8 infants cleared virus by 36 months, but none cleared virus before 15 months of age.

DISCUSSION

Our cohort of 245 HIV-coinfected GBV-C RNA–positive mothers and their infants is the largest single cohort of GBV-C–infected pregnant women examined to date and the first, to our knowledge, to report an association between MTCT of GBV-C and maternal ART. In our study, 41% of GBV-C–infected mothers transmitted GBV-C to their infants. This is similar to rates of MTCT of GBV-C observed in 3 smaller cohorts of HIV-coinfected women [23, 25, 26]. Maternal receipt of ART, high maternal GBV-C load, vaginal mode of delivery, and absence of infant HIV infection were independently associated with increased MTCT of GBV-C.

We observed that maternal receipt of ART increased the likelihood of MTCT of GBV-C. Of interest, Bjorkman and Widell [42], when reviewing our previous publication [22], noted that aggregate data presented in our manuscript pointed to an effect of ART on MTCT of GBV-C, as confirmed in the present manuscript. Because receipt of ART by neonates alone was not associated with increased rate of MTCT of GBV-C, any effect of ART appears to occur during pregnancy or at delivery. The addition of single-dose nevirapine (at delivery and to neonates) to zidovudine prophylaxis starting at 34–36 weeks of gestation also did not increase the rate of MTCT of GBV-C. There is mounting evidence that ART plays a role in both GBV-C and HIV replication and transmission. Highly active ART was associated with increased GBV-C titers and decreased HIV titers in HIV-coinfected patients in a recent study [43]; in our study, ART was associated with increased rate of MTCT of GBV-C and decreased rate of MTCT of HIV. However, maternal GBV-C load was not significantly associated with maternal receipt of ART (after adjusting for study; data not shown). Because ART is unlikely to have affected GBV-C replication directly (a flavivirus being quite different from a retrovirus), something associated with HIV inhibition, perhaps the reduction of HIV-induced systemic immune activation [43], may have resulted in enhanced replication and transmission of GBV-C. However, the inverse correlation that we observed between GBV-C and HIV load was quite weak [22], and the observed association between maternal receipt of ART and in-
creased rate of MTCT of GBV-C was independent of both maternal HIV and GBV-C load. Therefore, the biologic pathway for an effect of ART on MTCT of GBV-C remains unknown.

ART has been associated with a reduced rate of MTCT of HIV independent of its effect on reducing HIV load [44]. Our results may suggest another potential mechanism by which maternal receipt of ART prevents MTCT of HIV, because GBV-C infection may protect against HIV infection. In support of this, HIV infection of the infant was associated with a lower rate of MTCT of GBV-C, and coinfection in the infants occurred less frequently than would be predicted if they were independent events. In the combined study, only 2 (0.8%) of 245 infants of HIV–GBV-C–coinfected mothers acquired both GBV-C and HIV at birth, whereas with perinatal transmission rates of 13% for HIV and 41% for GBV-C in our study, we might have expected 13 (5%) of 245 infants to be coinfected. Two other studies also observed a low prevalence of GBV-C infection among HIV-infected children [45, 46]; however, we identified 3 studies of MTCT of GBV-C in HIV-infected women, and none of these studies observed a difference in cotransmission of HIV or GBV-C [23, 25, 26]. Possible reasons for the lack of association observed in these studies include small sample sizes ranging from 2 to 9 GBV-C–infected infants and different study populations. A mechanism for the strong inverse association between infant GBV-C and HIV infection that we observed remains unknown. Because both HIV and GBV-C infection appear to be vertically acquired by infants at approximately the same time [22], it is possible that either virus may inhibit the other or that a third factor (e.g., placental inflammation, which has been associated in some studies with reduced MTCT of HIV [47–49]) or the infant’s cytokine milieu prevents coinfection with both viruses. However, because GBV-C appears to reduce replication of HIV in vitro, most strongly when the cell is infected with GBV-C first, but also when the cell is infected simultaneously with HIV and GBV-C [17], there is a biologically plausible basis for the reduction of MTCT of HIV through infant GBV-C infection.

We confirmed observations from previous studies [1, 2, 11] that, not surprisingly, high GBV-C load and vaginal mode of delivery are associated with increased MTCT of GBV-C. Because the rate of MTCT of GBV-C was 67% at a GBV-C load of $\geq 8 \log_{10}$ copies/mL, there may not be a threshold over which transmission is 100%. However, both of the mothers with a GBV-C load of $9 \log_{10}$ viral copies/mL at delivery transmitted GBV-C to their infants (both also delivered vaginally). Although not significant, our data are consistent with elective caesarean deliveries having the lowest rate of MTCT of GBV-C, compared with other forms of delivery, as was reported in 2 other studies [1, 2].

Risk factors associated with increased viral exposure, such as high maternal viral load and a vaginal delivery, were similar for MTCT of HIV and MTCT of GBV-C. However, risk factors commonly associated with increased MTCT of HIV (no maternal receipt of ART, high maternal HIV load, low maternal CD4+ cell count, prematurity, and female sex of the infant) were reversed for MTCT of GBV-C. Mechanisms for this dichotomy are unknown.

The timing of GBV-C transmission is unknown, but because 21% of newborn samples available for testing were positive for GBV-C RNA, in utero transmission is likely for at least some infants. The remainder were likely infected either in utero or at delivery, given that 16 (94%) of 17 tested positive at 2 months and that mode of delivery was significantly associated with MTCT of GBV-C. Our study, in which all GBV-C RNA was of genotype 2, 3, or 4, supports evidence of vertical transmission of GBV-C; however, some studies from Africa, where genotypes 1 and 5 are most prevalent [50], have also reported evidence of horizontal transmission [24, 35].

Our study had several limitations. Because all study participants were HIV-infected Thai women, the generalizability of our findings may be limited to certain populations or GBV-C genotypes. A limitation of studies on GBV-C in general is the lack of a gold standard test for the detection of GBV-C RNA. Although reasons for the significantly lower maternal GBV-C titers in study 1 are unknown, possible explanations may include duration of specimen storage or changes in the population or epidemiology of GBV-C over the study periods. Studies of hepatitis C virus, the closest known relative to GBV-C, are suggestive of viral deterioration with long-term storage; however, the effect of duration of storage on the stability of the GBV-C has not been established. Among women who did not receive ART, the rate of MTCT of GBV-C was similar in all 3 perinatal studies (table 3). Because viral load is a major determinant of MTCT of HIV, this might indicate that the true maternal GBV-C load in study 1 was similar to that observed in studies 2 and 3. Differences in maternal GBV-C load appear to have confounded our estimate of study differences in the rate of MTCT of GBV-C. Although positive samples from infants were not confirmed with genotype testing, only 4 indeterminate samples from infants were excluded from analysis, and this would not have affected our results.

In summary, in our cohort of GBV-C–HIV-coinfected women, GBV-C was frequently transmitted from mother to infant. Maternal receipt of ART was independently associated with increased rate of MTCT of GBV-C, although the mechanism remains unknown. We observed that high maternal GBV-C load and vaginal mode of delivery—both factors that increase the level of virus to which an infant is exposed—were associated with increased rate of MTCT of GBV-C. Despite mode of de-
livery being a common risk factor, GBV-C and HIV were rarely cotransmitted.

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References


Chapter 5

Paper 3: Prevalence and correlates of GB virus C infection in HIV-infected and HIV-uninfected pregnant women in Bangkok, Thailand

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Prevalence and correlates of GB virus C infection in HIV-infected and HIV-uninfected pregnant women in Bangkok, Thailand

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ABSTRACT

Background: GB virus C (GBV-C) is an apathogenic virus that has been shown to inhibit HIV replication. Our study examined the prevalence and correlates of GBV-C infection and clearance in a large cohort of pregnant women in Thailand.

Methods: The study population consisted of 1,719 (1,387 HIV-infected and 332 HIV-uninfected) women from three Bangkok perinatal HIV transmission studies. Stored blood was tested for GBV-C RNA, antibody, and if RNA-positive, genotype. Risk factors associated with the prevalence of ever having GBV-C infection (defined as presence of GBV-C RNA and/or antibody) and viral clearance (defined as presence of GBV-C antibody in the absence of RNA) among women ever having GBV-C infection were examined using multiple logistic regression.
**Results:** The prevalence of ever having GBV-C infection was 33% among HIV-infected women and 15% among HIV-uninfected women. GBV-C infection was independently associated (AOR, 95%CI) with an increasing number of lifetime sexual partners, 2 partners (1.60, 1.22-2.08), 3 – 10 partners (1.92, 1.39-2.67), >10 partners (2.19, 1.33-3.62); injection drug use (5.50, 2.12-14.2); and HIV-infection (3.79, 2.58-5.59).

Clearance of GBV-C RNA among women with evidence of GBV-C infection was independently associated with increasing age (years) (reference <20, 20-29 (2.01, 1.06-3.79) and ≥30 (3.18, 1.53-6.60), more than 10 lifetime sexual partners (3.05, 1.38-6.75), and HIV coinfection (0.29, 0.14-0.59).

**Conclusions:** In Thailand, GBV-C infection is common among HIV-infected and HIV-uninfected women and is associated with HIV status as well as both sexual and parenteral risk behaviours. GBV-C viremia probably clears more slowly among HIV-coinfected women.
INTRODUCTION

GB virus C (GBV-C), a flavivirus closely related to hepatitis C virus (HCV) and not known to cause disease, may be important because of its potentially inhibitory effect on human immunodeficiency virus (HIV) replication. GBV-C has been observed to inhibit HIV \textit{in vitro} [Xiang et al., 2001] and, among HIV-infected persons, has been associated with improved survival in some studies [Heringlake et al., 1998; Lefrere et al., 1999b; Tillmann et al., 2001; Williams et al., 2004; Xiang et al., 2001; Yeo et al., 2000] while loss of GBV-C RNA has been associated with poor survival in longitudinal studies [Bjorkman et al., 2004; Van der Bij et al., 2005; Williams et al., 2004]. Mother-to-child-transmission (MTCT) of GBV-C has also been associated with reduced MTCT of HIV [Bhanich Supapol et al., 2008].

GBV-C RNA prevalence in the general population is higher than that of other blood-borne viruses such as hepatitis C and HIV in Western industrialized countries and varies globally. Among pregnant women, prevalence varies from 1-2\% in East Asia [Lin et al., 1998; Ohto et al., 2000], 5-7\% in Europe, Australia, and Southeast Asia [Hyland et al., 1998; Lefrere et al., 2000; Poovorawan et al., 1998; Skidmore and Collingham, 1999], and 10-13\% in Africa [Liu et al., 2000; Tuveri et al., 2000]. GBV-C genotype also differs by geographic location, and genotypes 3 and 4 were identified in Asia and Southeast Asia, respectively, genotypes 1 and 5 in West Africa and South Africa, respectively, and genotype 2 in North America and Europe [Tucker and Smuts, 2000]. A new genotype 6 was recently identified in Indonesia [Muerhoff et al., 2006]. GBV-C prevalence is higher among HIV-infected and HCV-infected persons and others with risk
factors for blood-borne infections [Stapleton et al., 2004] and some groups of injection
drug users (IDUs) and men who have sex with men (MSM) have evidence of nearly
universal GBV-C infection (80-90%) [Stapleton, 2003]. While GBV-C viremia may
persist for years, it is cleared eventually in 50-75% of persons, at which time antibodies
to the GBV-C E2 envelope usually appear [Kleinman, 2001]. There is some evidence
that HIV-infected persons and infants and children may clear GBV more slowly than
those with fully functioning immune systems [Chen et al., 1998; Devereux et al., 1998;
George et al., 2002; Hardikar et al., 1999]. Because antibody is not always detectable
after viral clearance [Bjorkman et al., 2004; Van der Bij et al., 2005; Williams et al.,
2004] and can deteriorate over time, possibly more rapidly among HIV-infected persons
[Devereux et al., 1998; Stark et al., 1999], it may be difficult to accurately estimate the
true prevalence GBV-C infection and associated relative risks when using an antibody-
based assay to determine infection.

Studies of the prevalence and correlates of GBV-C infection among different
populations have identified many risk factors for GBV-C infection, including, injection
drug use [Christensen et al., 2003; Katayama et al., 1997; Lefrere et al., 1999a; Saganuma
et al., 1998; Tan et al., 1999; Vanhems et al., 2003], receipt of blood transfusion
[Henrichsen et al., 2002; Stark et al., 1999; Tan et al., 1999], increased number of sexual
partners [Bjorkman et al., 2001; Ribeiro-dos-Santos et al., 2002; Wu et al., 1997], having
had sex with other men [Berzsenyi et al., 2005; Lefrere et al., 1999a; Rey et al., 2000;
Stark et al., 1999], snorting cocaine [Tan et al., 1999], imprisonment [Tan et al., 1999],


history of sexually transmitted infections (STIs) [Bjorkman et al., 2001; Tan et al., 1999],
health care work [Christensen et al., 2003], hospitalization for medical procedures such
as delivery and abortion [Lefrere et al., 1999a], endoscopy [Bjorkman et al., 2001], and
bronchoscopy [Vanhems et al., 2003], as well as a history of travel to Africa [Lefrere et
al., 1999a], schistosomiasis [Hassoba et al., 1997], and exposure to blood-sucking insects
[Ribeiro-dos-Santos et al., 2002]. In our study cohort of 1,387 HIV-infected and 332
HIV-uninfected pregnant women drawn from three perinatal studies in Bangkok, we
examined the associations of GBV-C RNA and antibody prevalence with demographic,
medical, and risk behaviour variables to determine risk factors for GBV-C infection and
clearance.

METHODS

The study population consisted of participants enrolled by March 31, 2004 in one
of three perinatal HIV transmission studies (hereafter “Peri-1”, “Peri-2”, and “Peri-3”) conducted jointly by the Thailand Ministry of Public Health and the U.S. Centers for Disease Control and Prevention (CDC) at two large Bangkok hospitals and for whom we were able to determine GBV-C infection status. Peri-1 (1992-94), a natural history study conducted prior to the advent of antiretroviral therapy (ART) for the prevention of MTCT of HIV, enrolled 342 HIV-infected women and a comparison group of 344 HIV-uninfected women [Shaffer et al., 1999b]. Peri-2 (1996-98; n=397) was a randomized, placebo-controlled trial assessing the efficacy of short-course zidovudine prophylaxis [Shaffer et al., 1999a]. Peri-3 (1999-2004; n=759) was an observational study assessing the implementation of programs to prevent MTCT of HIV [Teeraratkul et al., 2005]. Six
hundred and thirty (83%) who enrolled in Peri-3 received zidovudine prenatally or at
delivery including 220 (29%) who also received nevirapine.

Stored blood specimens from 1,783 (97%) of the 1,842 women enrolled in the
three studies were available and tested for GBV-C RNA, antibody and, if RNA-positive,
genotype. HIV-infected women had a delivery plasma specimen tested for evidence of
GBV-C infection while HIV-uninfected women had an enrolment serum sample tested.
Specimens were collected and tested for HIV as part of the original studies. HIV viral
load, CD4+, and CD8+ cell count were measured as previously described [Shaffer et al.,
1999a; Shaffer et al., 1999b]. Laboratory staff testing for GBV-C RNA and antibody
were blind to subject data.

GBV-C viral RNA was extracted from 200 μL of plasma using the QIAamp
MinElute Virus Vacuum kit (Qiagen Inc., Valencia, CA) and quantified using the
Quantitect Probe RT-PCR kit (Qiagen Inc., Valencia, CA) according to the
manufacturer’s instructions. The lower limit of reliable detection of this quantitative RT-
PCR test is 1,000 viral copies per reaction. Positive samples, including 55 with 1-999
viral copies, were confirmed by genotype analysis as previously described [Naito and
Abe, 2001; Schleicher and Flehmig, 2003]. Specimens were tested for antibody to the
GBV-C E2 envelope protein (anti-E2) by a μPLATE Anti-HGenv microtiter assay
(Roche Diagnostics, Indianapolis, IN).
We classified women into one of four mutually exclusive categories: 1) *GBV-C RNA positive* (with or without antibody): ≥1,000 viral copies detected with real-time RT-PCR reaction or 1-999 viral copies but able to genotype; 2) *GBV-C antibody positive*: anti-E2 serology positive and GBV-C RNA negative; 3) *GBV-C negative*: GBV-C RNA negative and anti-E2 serology negative; and 4) *GBV-C RNA indeterminate*: 1-999 copies of virus detected and unable to genotype.

Statistical analyses were performed with SAS version 8.2 (SAS Institute Inc., Cary, NC). We assessed the statistical significance of associations of demographic, socioeconomic, medical, and risk behaviour variables with ever having GBV-C infection within each study with two-sided p values from chi-square or Fisher’s exact tests, using tests for trend for ordered categorical variables. Homogeneity of odds ratios across studies was assessed with the Breslow-Day test. The three studies were combined for the multivariate analysis, with a variable indicating the original perinatal study included to adjust for possible unmeasured confounding between studies. Adjusted odds ratios (aORs) and 95% confidence intervals (CI) adjusted for Peri-1,-2, or -3 study participation were calculated using logistic regression. Variables available from all three studies for both HIV-infected and HIV-uninfected women that were associated with GBV-C infection in the analysis adjusted for study with a p value of less than 0.20 were included in the multivariate analysis and were retained in the final model if their p value was <0.05. If a linear relationship was not observed, continuous variables were categorized at generally used cut-points or at levels defined by the relationship observed with GBV-C infection. Logistic regression was also used to calculate aORs and 95% Wald CIs.
adjusted for multiple covariates. Interactions with HIV status were assessed by adding an
interaction term to the multivariable model where data permitted; an interaction could not
be assessed with IDU as there were no HIV-uninfected IDU. Similar methods were
employed in the analysis of correlates of antibody prevalence among those ever having
GBV-C infection.

This study was approved by the Research Ethics Board, University of Toronto,
Canada; the Institutional Review Board, CDC, Atlanta, U.S.; and the Ethical Review
Committees for Research in Human Subjects at the Thailand Ministry of Public Health
and Siriraj Hospital, Bangkok, Thailand.

RESULTS

GBV-C infection status was determined for 1,719 (96%) of 1,783 women; 64
(4%) women tested GBV-C RNA indeterminate and were excluded from further
analyses. Of women with known GBV-C status, 298 (17%) women had evidence of
active GBV-C infection (GBV-C RNA) including 17 (1%) who tested positive for both
GBV-C RNA and antibody; 212 (12%) had evidence of past GBV-C infection (GBV-C
antibody positive) and 1,209 (70%) tested negative for both RNA and antibody.
Characteristics of the 1,719 pregnant women with known GBV-C status are presented in
Table 1, by the perinatal study to which they originally enrolled and HIV status. More
HIV-infected women (459 [33%] of 1,387) had evidence of active or past GBV-C
infection (20% were GBV-C RNA-positive and 13% were antibody positive) compared
to HIV-uninfected (51 [15%] of 332) women (7% were GBV-C RNA-positive and 8%
were antibody positive); (p <0.0001). Among GBV-C infected women, GBV-C
genotypes 2 and 3 were most prevalent (39% and 35%, respectively) followed by genotype 4 (15%); 7% had multiple genotypes. Prevalence of GBV-C genotype 2 declined in our study population over the course of the three studies from 55% in Peri-1 to 28% in Peri-3, while genotypes 3 and 4 became more prevalent, from 28% and 9% respectively, to 42% and 21%. Prevalence of both GBV-C RNA and antibody were significantly higher among HIV-infected women compared to HIV-uninfected women (p<0.0001 and p=0.0009, respectively). The ratio of the number of women who were GBV-C viremic to the number of women with GBV-C antibody was 1.5 among HIV-infected women and 0.88 among HIV-uninfected women. Among HIV-infected women, prevalence of GBV-C RNA was higher in Peri-1 participants (33%) compared to Peri-2 (16%) and Peri-3 (17%) participants (p<0.0001) while the prevalence of GBV-C antibody was similar across the studies (Peri-1 12%, Peri-2 12%, and Peri-3 15%, p=0.35). GBV-C viral load was also lower in Peri-1 compared to Peri-2 and Peri-3 (p<0.0001) and GBV-C viral load was significantly lower among Peri-1 HIV-infected women compared to Peri-1 HIV-uninfected women (p=0.03). In addition to ART becoming more available, several other trends were observed over the study periods including that GBV-C genotypes 3 and 4 were more prevalent (p=0.001), that the women enrolled were slightly older (p<0.001) and more educated (p=0.01), and their lifetime number of sex partners was greater (p<0.0001). Most HIV-infected women in our study were young (median age 24 years) and in the early stages of HIV-infection (only 3% of women had a CD4+ count <200 cells/mm³, and 95% of Peri-1 and -2 women were WHO clinical stage 1 or less at enrolment [WHO stage not available for Peri-3]). HIV-infected women were more likely to have engaged in self or partner injection drug use (p=0.008 and <0.0001,
respectively), commercial sex work (CSW) \((p<0.001)\), and had had a higher lifetime number of sex partners \(<0.0001\).

Table 2 presents the associations with ever having GBV-C infection. In multivariate analysis, HIV infection, a history of injection drug use, and a higher lifetime number of sexual partners were independently associated with GBV-C infection after adjusting for study participation. The risk of GBV-C infection increased in a dose-response relationship with each category of lifetime number of sexual partners (1, 2, 3-10, and >10 partners). Most women (79%), reported either one or two lifetime sexual partners, and the increase from one to two sexual partners significantly increased the risk of GBV-C infection \((\text{AOR} \ 1.6, \ 95\% \ \text{CI} \ 1.22-2.08)\).

Other variables significantly associated with GBV-C infection that were not in the final multivariate model included: having commenced sexual activity at a younger age (by year), having had three or more pregnancies, having had CSW experience, self or partner having experienced intravenous drug use, self or partner having a history of sexually transmitted infections (STIs), having tattoos, having a husband who visited prostitutes, and women who were unmarried or who had less than a college education. CSW and younger age at first intercourse were not significantly associated with GBV-C infection when lifetime number of sex partners was included in the multivariate model. Because information regarding tattoos, husbands visiting prostitutes, and maternal or husband STIs were assessed in Peri-1 only, these variables could not be included in multivariate analysis. Having a low birthweight baby was also significantly associated
with ever having GBV-C infection in our study, but as birthweight was only available for children born to HIV-infected women, it was also excluded from the multivariate analysis.

Maternal age, hematocrit, family income, number of sex partners in the previous year, anal sex, number of people in a household, and prematurity of the infant were not associated with GBV-C infection after adjusting for study and, among HIV-infected women, maternal receipt of ART, HIV viral load, HIV subtype, CD4+ count and NK cell percent were not associated with GBV-C infection after adjusting for study (data not shown except maternal age and ART).

HIV status remained significantly associated with ever having GBV-C infection after controlling for many sexual risk behaviours (lifetime number of sex partners, CSW, and age at first intercourse) and parenteral risk behaviours (IDU, partner IDU, and transfusions). Of 18 women who thought that they had acquired HIV parenterally, 17 (94%) had evidence of GBV-C infection compared to 229 (28%) of 813 women who thought that they had acquired HIV sexually (Peri-2 and -3 data only). Interactions with HIV status were assessed where data permitted and none were significant.

Of 510 women with evidence of GBV-C infection (presence of GBV-C RNA or antibody), 212 (42%) had cleared the virus. For these 510 women, we examined predictors of clearing GBV-C (i.e. being antibody positive compared to RNA positive) in this cross-sectional analysis (Table 3). The 17 women with both RNA and antibody were
classified with the viremic group as not having cleared the virus. Significant predictors of being antibody positive adjusted for study included being HIV-uninfected, being 20 or more years of age, having had more than one pregnancy, and more than 10 lifetime sexual partners. HIV-infected women with an HIV viral load of less than 10,000 copies/mL were also significantly more likely to have cleared GBV-C. Maternal receipt of ART, CD4+ count, NK cell percent, a history of CSW, being an IDU, and age at first intercourse were not associated with presence of antibody. While interaction variables with HIV status were not significant, lifetime number of sex partners was associated with clearance only for HIV-infected women and only one HIV-negative woman had had more than 10 sex partners.

Among HIV-infected women in our study, GBV-C infection status was not associated with HIV viral load or CD4+ count (data not shown).

**DISCUSSION**

In our combined cohort of 1,719 pregnant women from three perinatal studies in Thailand, we observed a high rate of GBV-C infection (GBV-C RNA or antibody), 33% among HIV-infected women and 15% among HIV-uninfected women. The 20% prevalence of GBV-C RNA among HIV-infected women was similar to prevalence rates observed in several antenatal and other cohorts of HIV-infected persons [de Martino et al., 1998; Lefrere et al., 1999a; Lefrere et al., 1999b; Palomba et al., 1999; Tillmann et al., 2001] although some studies have observed rates as high as 40% [Williams et al., Feb. 22-25 2005; Xiang et al., 2001]. The 7% prevalence of GBV-C RNA among HIV-
uninfected women was similar to rates previously observed among pregnant women in Thailand [Poovorawan et al., 1998] and Europe [Lefrere et al., 2000; Skidmore and Collingham, 1999]. As we detected the presence of multiple GBV-C genotypes in 7% of women, infection with one GBV-C genotype may not prevent infection with another genotype; it is also possible that multiple genotypes could have been acquired simultaneously. The distribution of GBV-C genotypes we observed in Thailand among HIV-infected women included predominantly genotype 2, particularly in the first perinatal study. Although GBV-C genotypes 3 and 4 have been identified as the Asian and South-East Asian genotypes [Tucker and Smuts, 2000], these genotypes were relatively uncommon in the first perinatal study but increased in prevalence over the course of the three studies.

The higher prevalence of GBV-C infection among HIV-infected women is likely due to common parenteral and sexual routes of infection [Stapleton et al., 2004]. However, in our study, HIV-infection status remained significantly associated with GBV-C infection despite control for multiple established routes of acquiring HIV infection in the multivariate model. This was likely due to imperfect reporting and control of risk behaviours, as there is no evidence to support the belief that one virus predisposes to acquisition of the other [Bisson et al., 2005; Williams et al., 2005] except in the perinatal context [Bhanich Supapol et al., 2008]. However, it is also possible that other factors related to population structure or group dynamics might explain the higher prevalence of GBV-C infection among HIV-infected women.
Injection drug use was associated with a high risk of GBV-C infection. Since there were only 25 IDUs in our cohort (<2% of study subjects), all of whom were HIV-infected, this risk estimate may be somewhat unstable, but was similar to that observed in other cohorts [Christensen et al., 2003]. As injection drug use and having tattoos were associated with the highest risks of GBV-C infection, parenteral transmission may be more efficient than sexual transmission. However, sexual risk behaviours were more common than parenteral risks in our study population, and a high proportion of women in high sexual risk categories were also GBV-C infected, including commercial sex workers, women reporting more than 10 lifetime sexual partners, and women reporting first intercourse at less than 15 years of age. In addition, relatively low risk sexual activity, such as having two lifetime sexual partners, was significantly associated with GBV-C infection. In comparison, HCV infection among the same women was associated with parenteral risk factors only (IDU, partner IDU, and transfusions) and not sexual risk behaviour in multivariate analysis and the associations were much stronger than those observed for GBV-C [Jamieson et al., 2008]. Crowding, defined by the number of persons in a household, was not associated with GBV-C infection in our study, suggesting that household transmission of GBV-C through casual contact may be unlikely.

The ratio of GBV-C RNA to antibody prevalence was greater among HIV-infected women compared to HIV-uninfected women in all three perinatal studies, as also observed in previous studies [George et al., 2002; Lefrere et al., 1999a], suggesting that HIV-infected women may clear the virus more slowly than HIV-uninfected women.
Slower clearance of GBV-C among HIV-infected persons was observed in another study [Devereux et al., 1998; George et al., 2002] but not in one cohort of women in the HAART era, possibly due to stronger immune functioning after receiving HAART [Williams et al., 2005]. However, another explanation for this finding could be that HIV-infected women are less likely to develop or maintain antibodies to GBV-C. Several recent longitudinal studies have observed that as many as 75 to 80% of HIV-infected persons clear GBV-C viremia without antibody being detectable at a median of four to seven years later [Bjorkman et al., 2004; Van der Bij et al., 2005; Williams et al., 2004]; Stark et al. also observed that loss of antibody over time was higher among HIV-infected individuals [Stark et al., 1999].

Older age was associated with GBV-C clearance in our study as previously observed [Feucht et al., 1999; Rey et al., 2000] and is likely a function of duration of infection. Duration of infection may also explain the observed association of antibody prevalence with more than 10 lifetime sexual partners. HIV-negative serostatus may be associated with an increased likelihood of GBV-C clearance because HIV infected persons clear GBV-C more slowly as has been hypothesized [Kleinman, 2001], because HIV-infected persons do not develop or maintain antibodies as well as those with fully functioning immune systems, or both. Clearance of GBV-C RNA was associated in several longitudinal studies with poor survival; however most of these persons did not have detectable antibody at the time of measurement. In our cross-sectional study, where we did not directly observe viral clearance, presence of GBV-C antibody, which may
indicate better immune function, was associated with lower HIV viral load but not with
CD4+ count among HIV-infected women.

HIV antiretroviral therapy, which has been associated with increased GBV-C viral load [Bjorkman et al., 2007] and increased mother-to-child-transmission of GBV-C, [Bhanich Supapol et al., 2009] was not associated with presence of GBV-C infection or clearance in our cohort of pregnant women. Also, presence of GBV-C RNA was not associated with HIV viral load or CD4+ count in our study, possibly due to the relatively young age and early stage of HIV infection of these women. While GBV-C viral loads were significantly higher in HIV-negative women, suggesting that GBV-C replication may be higher in the absence of HIV replication, as all samples from HIV-infected women were plasma and all samples from HIV-uninfected women were serum, it is not possible to distinguish whether GBV-C viral loads were truly lower among HIV-infected women or whether something related to the plasma specimens, such as presence of heparin, might have reduced viral load or viral load detection.

Our study had several limitations. A limitation of GBV-C studies in general is the lack of a gold standard test for the detection of GBV-C RNA. Sexual and illicit drug use risk factors are often underreported. Because women with these risk factors are more likely to be GBV-C infected, underreporting would have biased the associations to the null. Associations for these variables may therefore be stronger than we observed. As this study was cross-sectional and antibody can disappear over time, some previously infected women may have been misclassified as negative. If misclassification were non-
differential with respect to covariates, then bias would have tended towards the null and some associations with GBV-C infection may not have been detected. If misclassification were differential, for example, if HIV-infected women were less likely to have detectable antibody, then it could have appeared that HIV-infected women had lower rates of GBV-C infection than they actually do and, that they were less likely to clear GBV-C. The use of prevalence to estimate causal associations may bias some estimates. For example, if women with GBV-C infection survive longer than women without GBV-C infection among HIV-infected women but not HIV-uninfected women, GBV-C infection may have appeared more common among HIV-infected women due to a survival bias. While a survival bias is unlikely given the relatively young age and early stage of HIV-infection of women in our study, prevalence rate ratios also tend to underestimate the underlying incidence rate ratio [Szklo and Nieto, 2000].

In conclusion, we observed a much higher prevalence of GBV-C infection among HIV-infected compared to HIV-uninfected pregnant women in Thailand likely due to common risk factors. Our study confirmed that parenteral risk factors, although not common in our cohort, were strongly associated with GBV-C infection as were measures of sexual risk behaviour. Clearance of GBV-C viremia among persons with detectable GBV-C infection appears to be associated with duration of infection and HIV-negative serostatus.
Table 1: Clinical and demographic characteristics of pregnant women included in the GBV-C study, according to the perinatal study in which they originally participated, Bangkok, Thailand (n=1,719)

<table>
<thead>
<tr>
<th>GBV-C status *</th>
<th>Peri-1 HIV- n=332</th>
<th>Peri-1 HIV+ n=280</th>
<th>Peri-2 HIV+ n=394</th>
<th>Peri-3 HIV+ n=713</th>
<th>Total HIV+ n=1,387</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA-positive</td>
<td>24 (7%)</td>
<td>93 (33%)</td>
<td>62 (16%)</td>
<td>119 (17%)</td>
<td>274 (20%)</td>
</tr>
<tr>
<td>Antibody positive</td>
<td>27 (8%)</td>
<td>33 (12%)</td>
<td>47 (12%)</td>
<td>105 (15%)</td>
<td>185 (13%)</td>
</tr>
<tr>
<td>Negative</td>
<td>281 (85%)</td>
<td>154 (55%)</td>
<td>285 (72%)</td>
<td>489 (69%)</td>
<td>928 (67%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GBV-C infection, active or past (RNA or antibody)</th>
<th>Peri-1 HIV- n=332</th>
<th>Peri-1 HIV+ n=280</th>
<th>Peri-2 HIV+ n=394</th>
<th>Peri-3 HIV+ n=713</th>
<th>Total HIV+ n=1,387</th>
</tr>
</thead>
<tbody>
<tr>
<td>51 (15%)</td>
<td>126 (45%)</td>
<td>109 (28%)</td>
<td>224 (31%)</td>
<td>459 (33%)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GBV-C viral load (log_{10} copies/mL) † median (IQR)</th>
<th>Peri-1 HIV- n=332</th>
<th>Peri-1 HIV+ n=280</th>
<th>Peri-2 HIV+ n=394</th>
<th>Peri-3 HIV+ n=713</th>
<th>Total HIV+ n=1,387</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.6 (4.5-7.9)</td>
<td>5.4 (4.1-6.6)</td>
<td>8.0 (7.5-8.4)</td>
<td>8.0 (6.8-8.3)</td>
<td>7.4 (5.7-8.1)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GBV-C genotype †</th>
<th>Peri-1 HIV- n=332</th>
<th>Peri-1 HIV+ n=280</th>
<th>Peri-2 HIV+ n=394</th>
<th>Peri-3 HIV+ n=713</th>
<th>Total HIV+ n=1,387</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>8 (33%)</td>
<td>51 (55%)</td>
<td>26 (42%)</td>
<td>32 (28%)</td>
<td>107 (40%)</td>
</tr>
<tr>
<td>2B</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (3%)</td>
<td>3 (3%)</td>
<td>5 (2%)</td>
</tr>
<tr>
<td>3</td>
<td>10 (42%)</td>
<td>26 (28%)</td>
<td>21 (34%)</td>
<td>48 (42%)</td>
<td>95 (35%)</td>
</tr>
<tr>
<td>4</td>
<td>2 (8%)</td>
<td>8 (9%)</td>
<td>11 (18%)</td>
<td>24 (21%)</td>
<td>43 (16%)</td>
</tr>
<tr>
<td>Multiple</td>
<td>4 (17%)</td>
<td>8 (9%)</td>
<td>2 (3%)</td>
<td>6 (5%)</td>
<td>18 (7%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age (years) median (IQR)</th>
<th>Peri-1 HIV- n=332</th>
<th>Peri-1 HIV+ n=280</th>
<th>Peri-2 HIV+ n=394</th>
<th>Peri-3 HIV+ n=713</th>
<th>Total HIV+ n=1,387</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 (21-28)</td>
<td>22 (20-26)</td>
<td>24 (22-28)</td>
<td>26 (23-30)</td>
<td>25 (22-28)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Education</th>
<th>Peri-1 HIV- n=332</th>
<th>Peri-1 HIV+ n=280</th>
<th>Peri-2 HIV+ n=394</th>
<th>Peri-3 HIV+ n=713</th>
<th>Total HIV+ n=1,387</th>
</tr>
</thead>
<tbody>
<tr>
<td>primary or less</td>
<td>221 (67%)</td>
<td>188 (67%)</td>
<td>229 (58%)</td>
<td>403 (57%)</td>
<td>820 (59%)</td>
</tr>
<tr>
<td>greater than primary</td>
<td>111 (33%)</td>
<td>92 (32%)</td>
<td>165 (42%)</td>
<td>310 (43%)</td>
<td>567 (43%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Place of birth</th>
<th>Peri-1 HIV- n=332</th>
<th>Peri-1 HIV+ n=280</th>
<th>Peri-2 HIV+ n=394</th>
<th>Peri-3 HIV+ n=713</th>
<th>Total HIV+ n=1,387</th>
</tr>
</thead>
<tbody>
<tr>
<td>rural</td>
<td>149 (45%)</td>
<td>151 (54%)</td>
<td>187 (47%)</td>
<td>360 (50%)</td>
<td>698 (50%)</td>
</tr>
<tr>
<td>urban</td>
<td>183 (55%)</td>
<td>129 (46%)</td>
<td>207 (53%)</td>
<td>353 (50%)</td>
<td>689 (50%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Occupational skill level‡</th>
<th>Peri-1 HIV- n=332</th>
<th>Peri-1 HIV+ n=280</th>
<th>Peri-2 HIV+ n=394</th>
<th>Peri-3 HIV+ n=713</th>
<th>Total HIV+ n=1,387</th>
</tr>
</thead>
<tbody>
<tr>
<td>skilled</td>
<td>9 (3%)</td>
<td>1 (&lt;1%)</td>
<td>3 (1%)</td>
<td>8 (1%)</td>
<td>12 (1%)</td>
</tr>
<tr>
<td>semi-skilled</td>
<td>64 (19%)</td>
<td>49 (18%)</td>
<td>108 (30%)</td>
<td>139 (22%)</td>
<td>296 (23%)</td>
</tr>
<tr>
<td>unskilled</td>
<td>259 (78%)</td>
<td>230 (82%)</td>
<td>253 (70%)</td>
<td>493 (77%)</td>
<td>976 (76%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total family monthly income (baht)‡</th>
<th>Peri-1 HIV- n=332</th>
<th>Peri-1 HIV+ n=280</th>
<th>Peri-2 HIV+ n=394</th>
<th>Peri-3 HIV+ n=713</th>
<th>Total HIV+ n=1,387</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 5,000 ($140)</td>
<td>54 (16%)</td>
<td>65 (25%)</td>
<td>38 (10%)</td>
<td>159 (24%)</td>
<td>262 (20%)</td>
</tr>
<tr>
<td>5,000-14,999 ($140-$425)</td>
<td>218 (66%)</td>
<td>151 (57%)</td>
<td>203 (56%)</td>
<td>376 (57%)</td>
<td>730 (56%)</td>
</tr>
<tr>
<td>≥15,000 ($425)</td>
<td>57 (17%)</td>
<td>49 (19%)</td>
<td>125 (34%)</td>
<td>131 (20%)</td>
<td>305 (24%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gravida</th>
<th>Peri-1 HIV- n=332</th>
<th>Peri-1 HIV+ n=280</th>
<th>Peri-2 HIV+ n=394</th>
<th>Peri-3 HIV+ n=713</th>
<th>Total HIV+ n=1,387</th>
</tr>
</thead>
<tbody>
<tr>
<td>median (IQR)</td>
<td>2 (1-2)</td>
<td>1 (1-2)</td>
<td>2 (1-2)</td>
<td>2 (1-2)</td>
<td>2 (1-2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Married §</th>
<th>Peri-1 HIV- n=332</th>
<th>Peri-1 HIV+ n=280</th>
<th>Peri-2 HIV+ n=394</th>
<th>Peri-3 HIV+ n=713</th>
<th>Total HIV+ n=1,387</th>
</tr>
</thead>
<tbody>
<tr>
<td>171 (52%)</td>
<td>168 (60%)</td>
<td>257 (65%)</td>
<td>344 (48%)</td>
<td>769 (55%)</td>
<td></td>
</tr>
<tr>
<td>Antiretroviral regimen \¶</td>
<td>\¶ no therapy</td>
<td>maternal ZDV</td>
<td>maternal and infant ZDV</td>
<td>infant only ZDV</td>
<td>mother and infant ZDV+ NVP</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------</td>
<td>---------------</td>
<td>------------------------</td>
<td>-----------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td></td>
<td>280 (100%)</td>
<td>198 (50%)</td>
<td>196 (50%)</td>
<td>381 (53%)</td>
<td>124 (17%)</td>
</tr>
<tr>
<td></td>
<td>196 (14%)</td>
<td>124 (17%)</td>
<td>124 (9%)</td>
<td>208 (29%)</td>
<td>208 (15%)</td>
</tr>
<tr>
<td>Maternal HIV viral load (log_{10} copies/mL) median (IQR) \¶</td>
<td>n=267 4.3</td>
<td>n=390 4.2</td>
<td>n=710 3.8</td>
<td>n=196 3.2-4.4</td>
<td>n=208 3.5-4.6</td>
</tr>
<tr>
<td>Maternal CD4 count (cells/mm³) median (IQR) \¶</td>
<td>n=276 450 330-570</td>
<td>n=390 380 267-529</td>
<td>n=711 403 271-548</td>
<td>n=208 280-545</td>
<td></td>
</tr>
<tr>
<td>Commercial sex worker</td>
<td>6 (2%)</td>
<td>30 (11%)</td>
<td>38/392 (10%)</td>
<td>60 (8%)</td>
<td>128 (9%)</td>
</tr>
<tr>
<td>Injection drug user (IDU) ‡</td>
<td>0 (0%)</td>
<td>3 (1%)</td>
<td>5 (1%)</td>
<td>17 (2%)</td>
<td>25 (2%)</td>
</tr>
<tr>
<td>Partner IDU ‡</td>
<td>5/330 (2%)</td>
<td>20/267 (7%)</td>
<td>38/361 (11%)</td>
<td>102/613 (17%)</td>
<td>160/1,241 (13%)</td>
</tr>
<tr>
<td>HIV subtype ‡¶</td>
<td>n=273 260 95%</td>
<td>n=362 322 89%</td>
<td>n=605 536 89%</td>
<td>n=1,240 1,118 90%</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>10 (4%)</td>
<td>22 (6%)</td>
<td>44 (7%)</td>
<td>76 (6%)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>3 (1%)</td>
<td>16 (4%)</td>
<td>11 (2%)</td>
<td>30 (2%)</td>
<td></td>
</tr>
<tr>
<td>E/B</td>
<td>0 (0%)</td>
<td>2 (1%)</td>
<td>14 (2%)</td>
<td>16 (1%)</td>
<td></td>
</tr>
<tr>
<td>BR/MN/C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood transfusion (1985-89) ‡</td>
<td>4/329 (1%)</td>
<td>3/273 (1%)</td>
<td>10/379 (3%)</td>
<td>13/625 (2%)</td>
<td>26/1,277 (2%)</td>
</tr>
<tr>
<td>Age at first intercourse median (IQR)</td>
<td>19 17-22</td>
<td>19 17-21</td>
<td>19 17-22</td>
<td>18 17-20</td>
<td></td>
</tr>
<tr>
<td>Lifetime number of sexual partners</td>
<td>n=278 252 (76%)</td>
<td>n=394 139 (50%)</td>
<td>n=706 157 (39%)</td>
<td>n=1,376 450 (33%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>68 (20%)</td>
<td>90 (32%)</td>
<td>158 (40%)</td>
<td>331 (47%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>32 (12%)</td>
<td>58 (15%)</td>
<td>180 (26%)</td>
<td>270 (20%)</td>
<td></td>
</tr>
<tr>
<td>3-10</td>
<td>17 (6%)</td>
<td>22 (6%)</td>
<td>38 (5%)</td>
<td>77 (6%)</td>
<td></td>
</tr>
<tr>
<td>11-9000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*indeterminate excluded from analysis
† GBV-C RNA-positive women only
‡ denominator provided where data missing
§ marriage definition: Peri 1 married vs. living together or single; Peri-2 and -3 married traditional and legal vs single with relationship or with no relationship
¶ HIV-infected women only
Table 2 Risk factors for GBV-C infection (presence of GBV-C RNA and/or antibody) in 1,719 HIV-infected and HIV-uninfected pregnant women, Bangkok, Thailand

<table>
<thead>
<tr>
<th>Variable</th>
<th>GBV-C infected women (RNA or anti-E2) (n/N, %)</th>
<th>Odds of maternal GBV-C infection adjusted for study</th>
<th>Odds of maternal GBV-C infection adjusted for study and covariates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AOR</td>
<td>95% CI</td>
</tr>
<tr>
<td>HIV status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>459/1,387</td>
<td>33%</td>
<td>4.51</td>
</tr>
<tr>
<td>Not infected</td>
<td>51/332</td>
<td>15%</td>
<td>1.0</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>64/204</td>
<td>trend p=0.79</td>
<td>1.0</td>
</tr>
<tr>
<td>20-39</td>
<td>350/1,198</td>
<td>29%</td>
<td>0.91</td>
</tr>
<tr>
<td>≥30</td>
<td>96/317</td>
<td>30%</td>
<td></td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>College/University</td>
<td>18/96</td>
<td>trend p=0.003</td>
<td>0.37</td>
</tr>
<tr>
<td>Primary, Junior, High</td>
<td>458/1,535</td>
<td>29%</td>
<td>0.69</td>
</tr>
<tr>
<td>Less than primary</td>
<td>34/87</td>
<td>39%</td>
<td>1.0</td>
</tr>
<tr>
<td>Married †</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>256/940</td>
<td>27%</td>
<td>0.79</td>
</tr>
<tr>
<td>No</td>
<td>254/779</td>
<td>33%</td>
<td>1.0</td>
</tr>
<tr>
<td>Gravida</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3+ pregnancies</td>
<td>138/391</td>
<td>35%</td>
<td>1.38</td>
</tr>
<tr>
<td>1-2 pregnancies</td>
<td>371/1326</td>
<td>28%</td>
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</tr>
<tr>
<td>Maternal antiretroviral therapy †</td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>233/785</td>
<td>30%</td>
<td>0.86</td>
</tr>
<tr>
<td>No</td>
<td>277/934</td>
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<td>Lifetime number of sexual partners</td>
<td></td>
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<tr>
<td>1</td>
<td>153/702</td>
<td>trend p=0.0001</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>208/647</td>
<td>22%</td>
<td>1.82</td>
</tr>
<tr>
<td>3-10</td>
<td>105/280</td>
<td>32%</td>
<td>2.34</td>
</tr>
<tr>
<td>11-9000</td>
<td>34/79</td>
<td>38%</td>
<td>2.91</td>
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<td>Age at first intercourse (years)</td>
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<tr>
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<td>29/74</td>
<td>trend p=0.01</td>
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</tr>
<tr>
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<td>308/1003</td>
<td>39%</td>
<td>0.95 per year increase in age</td>
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<tr>
<td>20-24</td>
<td>138/496</td>
<td>31%</td>
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</tr>
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<td>25-29</td>
<td>31/125</td>
<td>28%</td>
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<td>30+</td>
<td>4/21</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Study 1</td>
<td>Study 2</td>
<td>Study 3</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Commercial sex worker</td>
<td>63/134</td>
<td>19/25</td>
<td>134/165</td>
</tr>
<tr>
<td></td>
<td>447/1583</td>
<td>491/1694</td>
<td>399/1406</td>
</tr>
<tr>
<td></td>
<td>47%</td>
<td>76%</td>
<td>39%</td>
</tr>
<tr>
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<td>28%</td>
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<td>28%</td>
</tr>
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<td>1.0</td>
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<td>3.01-19.17</td>
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<td>0.01</td>
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<tr>
<td>Injection drug user (IDU)</td>
<td>19/25</td>
<td>64/165</td>
<td>7/12</td>
</tr>
<tr>
<td></td>
<td>491/1694</td>
<td>399/1406</td>
<td>170/600</td>
</tr>
<tr>
<td></td>
<td>76%</td>
<td>39%</td>
<td>58%</td>
</tr>
<tr>
<td></td>
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<td>28%</td>
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<td>1.35</td>
<td>1.55</td>
<td>3.54</td>
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<td></td>
<td></td>
<td>1.0</td>
<td>1.0</td>
</tr>
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<td></td>
<td>1.02-1.79</td>
<td>1.10-2.18</td>
<td>1.11-11.31</td>
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<td>&lt;0.0001</td>
<td>0.03</td>
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<td>Any partner IDU</td>
<td>63/134</td>
<td>13/32</td>
<td>7/12</td>
</tr>
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<td></td>
<td>447/1583</td>
<td>497/1683</td>
<td>170/600</td>
</tr>
<tr>
<td></td>
<td>47%</td>
<td>41%</td>
<td>58%</td>
</tr>
<tr>
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<td>30%</td>
<td>28%</td>
</tr>
<tr>
<td></td>
<td>1.35</td>
<td>1.63</td>
<td>3.54</td>
</tr>
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<td>1.0</td>
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<td>0.80-3.34</td>
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<td>N/I</td>
</tr>
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<td>Received blood transfusion (1985-89)</td>
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<td>134/391</td>
<td>83/196</td>
</tr>
<tr>
<td></td>
<td>497/1683</td>
<td>33/178</td>
<td>72/348</td>
</tr>
<tr>
<td></td>
<td>41%</td>
<td>34%</td>
<td>42%</td>
</tr>
<tr>
<td></td>
<td>30%</td>
<td>19%</td>
<td>21%</td>
</tr>
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<td></td>
<td>1.35</td>
<td>2.29</td>
<td>2.82</td>
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<td></td>
<td>1.0</td>
<td>1.0</td>
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<td>1.48-3.53</td>
<td>1.92-4.13</td>
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<td>&lt;0.0001</td>
<td>N/I</td>
</tr>
<tr>
<td>Husband visits prostitutes (Peri-1 only)</td>
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<td>83/196</td>
<td>Peri-1 only</td>
</tr>
<tr>
<td></td>
<td>33/178</td>
<td>72/348</td>
<td>37/78</td>
</tr>
<tr>
<td></td>
<td>399/1406</td>
<td>499/1694</td>
<td>138/526</td>
</tr>
<tr>
<td></td>
<td>47%</td>
<td>42%</td>
<td>47%</td>
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<tr>
<td></td>
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<td>21%</td>
<td>21%</td>
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<td>2.82</td>
<td>2.54</td>
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<td></td>
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<td>1.0</td>
</tr>
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<td></td>
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<td>1.48-3.53</td>
<td>1.56-4.12</td>
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<tr>
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<td>0.04</td>
<td>&lt;0.0001</td>
<td>N/I</td>
</tr>
<tr>
<td>Husband had STIs (Peri-1 only)</td>
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<td>83/196</td>
<td>Peri-1 only</td>
</tr>
<tr>
<td></td>
<td>33/178</td>
<td>72/348</td>
<td>37/78</td>
</tr>
<tr>
<td></td>
<td>399/1406</td>
<td>499/1694</td>
<td>138/526</td>
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<tr>
<td></td>
<td>47%</td>
<td>42%</td>
<td>47%</td>
</tr>
<tr>
<td></td>
<td>26%</td>
<td>21%</td>
<td>21%</td>
</tr>
<tr>
<td></td>
<td>1.35</td>
<td>2.82</td>
<td>2.54</td>
</tr>
<tr>
<td></td>
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<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
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<td>1.48-3.53</td>
<td>1.56-4.12</td>
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<tr>
<td></td>
<td>0.04</td>
<td>&lt;0.0001</td>
<td>N/I</td>
</tr>
<tr>
<td>Ever had an STI (Peri-1 only)</td>
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<td>83/196</td>
<td>Peri-1 only</td>
</tr>
<tr>
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<td>399/1248</td>
<td>72/348</td>
<td>37/78</td>
</tr>
<tr>
<td></td>
<td>43%</td>
<td>42%</td>
<td>47%</td>
</tr>
<tr>
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<td>21%</td>
<td>21%</td>
</tr>
<tr>
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<td>1.35</td>
<td>2.82</td>
<td>2.54</td>
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<tr>
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<td></td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1.02-1.79</td>
<td>1.48-3.53</td>
<td>1.56-4.12</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>&lt;0.0001</td>
<td>N/I</td>
</tr>
<tr>
<td>Low birthweight (&lt;2,500 gm) †</td>
<td>60/139</td>
<td>83/196</td>
<td>Peri-1 only</td>
</tr>
<tr>
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<td>399/1248</td>
<td>72/348</td>
<td>37/78</td>
</tr>
<tr>
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<td>42%</td>
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<td>21%</td>
<td>21%</td>
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<td>2.82</td>
<td>2.54</td>
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<td></td>
<td></td>
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<td>1.0</td>
</tr>
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<td>1.48-3.53</td>
<td>1.56-4.12</td>
</tr>
<tr>
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<td>&lt;0.0001</td>
<td>N/I</td>
</tr>
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<td>109/394</td>
<td>224/713</td>
</tr>
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<td>31%</td>
</tr>
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<td>0.94</td>
<td>1.13</td>
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<td>0.71-1.25</td>
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<td>0.44</td>
<td>0.32</td>
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<tr>
<td></td>
<td>0.44</td>
<td>0.32-0.61</td>
<td>0.35-0.64</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*married defined slightly differently between studies: Peri 1, married vs. living together and single; Peri-2&3, married traditional & legal vs single with relationship and single with no relationship
† HIV+ only
N/I – not included in multivariate regression
Table 3: Correlates of clearing GBV-C infection among 510 Thai women with evidence of GBV-C infection

<table>
<thead>
<tr>
<th>Variable</th>
<th>(n/N, %) of women who cleared GBV-C</th>
<th>OR adjusted for study only</th>
<th>Final multivariate model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AOR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td>trend p &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>16/64</td>
<td>1.0</td>
<td>1.07-3.62</td>
</tr>
<tr>
<td>20-29</td>
<td>143/350</td>
<td>1.97</td>
<td>1.63-6.69</td>
</tr>
<tr>
<td>≥30</td>
<td>53/96</td>
<td>3.31</td>
<td>2.01-3.10</td>
</tr>
<tr>
<td>HIV status</td>
<td></td>
<td>trend p &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>185/459</td>
<td>0.32</td>
<td>0.16-0.62</td>
</tr>
<tr>
<td>Not infected</td>
<td>27/51</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Lifetime number of sexual partners</td>
<td></td>
<td>trend p &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>52/153</td>
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<td></td>
</tr>
<tr>
<td>2-10</td>
<td>134/313</td>
<td>1.28</td>
<td>0.80-1.88</td>
</tr>
<tr>
<td>&gt;10</td>
<td>20/34</td>
<td>2.57</td>
<td>1.87-6.75</td>
</tr>
<tr>
<td>Gravida</td>
<td></td>
<td>trend p &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>&gt;1 pregnancy</td>
<td>141/298</td>
<td>1.73</td>
<td>1.20-2.50</td>
</tr>
<tr>
<td>1 pregnancy</td>
<td>70/211</td>
<td>1.0</td>
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<td>Maternal antiretroviral therapy *†</td>
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<td>trend p &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>102/233</td>
<td>0.73</td>
<td>0.44-1.19</td>
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<tr>
<td>No</td>
<td>110/277</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>HIV viral copies/mL†</td>
<td></td>
<td>trend p &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>≥10,000</td>
<td>81/244</td>
<td>0.57</td>
<td>0.38-0.84</td>
</tr>
<tr>
<td>&lt;10,000</td>
<td>101/207</td>
<td>1.0</td>
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</tr>
<tr>
<td>CD4+ cell count †</td>
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<td>trend p &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>&lt;200 cells/mm³</td>
<td>16/46</td>
<td>0.67</td>
<td>0.35-1.28</td>
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<tr>
<td>≥200 cells/mm³</td>
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</tr>
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<td>Low birth weight (&lt;2500 gms) †</td>
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<td>trend p &lt;0.0001</td>
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</tr>
<tr>
<td>Commercial sex worker</td>
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<td>trend p &lt;0.0001</td>
<td></td>
</tr>
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<tr>
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<td>183/447</td>
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</tr>
<tr>
<td>Injection drug user</td>
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<td>trend p &lt;0.0001</td>
<td></td>
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<td>11/19</td>
<td>1.41</td>
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<tr>
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<td>201/491</td>
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</tr>
<tr>
<td>Study †</td>
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<td>trend p &lt;0.0001</td>
<td></td>
</tr>
<tr>
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<tr>
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<td>Study 3</td>
<td>105/224</td>
<td>1.72</td>
<td>1.15-2.59</td>
</tr>
</tbody>
</table>
*ART – crude proportion showing women receiving ART were more likely to have antibody is misleading. In Peri-2 and 3 where ART was available, prevalence of antibody was less among women receiving ART, although not significantly so.
† HIV-infected women only
‡ prevalence of GBV-C RNA was higher in Peri-1 it makes it appear as though clearance was infrequent
N/I - not included in multivariate regressions as have data for HIV+ only
References


virus type C in the women's interagency HIV study [abstract 942]; Feb. 22-25 2005; Boston, MA, USA.


Chapter 6
Discussion

The discussion comprises of three main parts: a summary of the results including contribution to knowledge regarding the interaction between HIV and GBV-C, strengths and limitations, and future research directions.

6.1 SUMMARY OF RESULTS

6.1.1 OBJECTIVE 1: TO DETERMINE IF MATERNAL OR INFANT GBV-C INFECTION WAS ASSOCIATED WITH REDUCED MTCT OF HIV

In our large study of 1,364 HIV-infected pregnant women, we observed that infant but not maternal GBV-C was associated with reduced mother-to-child transmission of HIV. This surprise finding was observed in all three perinatal studies (although only significant in Peri-1, all were in the same direction and the overall effect was very strong). Because HIV and GBV-C are likely acquired at similar times, the direction of causation is difficult to establish and the location and nature of the interaction between HIV and GBV-C in the perinatal context remains unknown.

We had expected that maternal GBV-C infection might reduce MTCT of HIV through decreased HIV replication but we observed no protective effect of maternal...
GBV-C infection. We can confirm in our large study, similar observations from two smaller studies published after we began our study [Handelsman et al., 2007; Weintrob et al., 2004], although the former did observe a borderline protective effect for women receiving HAART.

Given the strength of the infant GBV-C association and that infants can only acquire GBV-C from their mothers, we might have expected that maternal GBV-C might also be significantly associated with MTCT of HIV, although as the rate of MTCT of GBV-C was only 41%, a smaller effect size would have been expected. To aid our understanding of this phenomenon we stratified our results by both maternal and infant GBV-C status (Appendix 4, Table A4.1). In Peri-2, the rate of MTCT of HIV was unexpectedly high among infants who did not acquire GBV-C from their GBV-C RNA-positive mothers, compared to infants whose mothers tested GBV-C RNA negative (OR 3.8, 95% CI 1.5-9.1). This association was observed in both the placebo and AZT arms of the trial, but was not observed in Peri-1 or Peri-3. Although Peri-2 had the smallest number of infants tested, these results were influential. Overall, among GBV-C RNA-negative infants, 20% of infants born to GBV-C RNA-positive mothers were HIV-infected compared to 13% of infants born to GBV-C negative mothers (OR 1.7, 95% CI 1.1-2.7), making it appear as though if a woman did not transmit GBV-C to her infant, that infant was at higher risk of acquiring HIV than if the mother had been GBV-C negative. This is somewhat analogous to results of longitudinal studies where those who lost GBV-C RNA over time were observed to be at a higher risk of disease progression than those who were never infected with GBV-C. It is not known why this phenomenon
was observed in only one perinatal study and not the others. In Peri-1 and Peri-3, while presence of maternal GBV-C RNA was not significantly associated with reduced MTCT of HIV, it was in the direction of a protective effect (Chapter 3, Table 2: OR 0.83 and 0.79, respectively). (Our study had the power to detect a protective odds ratio of 0.5 or higher). As all studies did not reject the null, the 95% confidence intervals overlapped, and a test of heterogeneity was not significant, we cannot conclude that there were study differences in the association between maternal GBV-C and MTCT of HIV. The location and nature of the interaction between GBV-C and HIV in the perinatal context remains unknown.

6.1.2 OBJECTIVE 2: TO DETERMINE THE RATE AND CORRELATES OF MOTHER-TO-CHILD TRANSMISSION OF GBV-C

In our study, 101 (41%) of 245 infants acquired GBV-C infection. MTCT of GBV-C was independently associated with maternal receipt of antiretroviral therapy, maternal GBV-C viral load in a dose-response relationship, a vaginal delivery, and absence of infant HIV infection, adjusting for study. Our study is the first to observe an association between ART and MTCT of GBV-C. This interesting finding could suggest that something related to HIV replication influences MTCT of GBV-C; however, maternal receipt of ART remained significantly associated with MTCT of GBV-C after adjusting for maternal HIV viral load. This finding may also explain why maternal GBV-C infection was observed to be protective against MTCT of HIV (although not at the 0.05 level of statistical significance) only in women receiving HAART; as GBV-C
RNA-positive women taking antiretrovirals are more likely to transmit GBV-C to their infants than women not taking antiretrovirals, and infant GBV-C acquisition was observed to be inversely associated with MTCT of HIV. We were able to characterize the highly dose dependent relationship of MTCT of GBV-C with GBV-C viral load and confirmed the role of maternal GBV-C viral load and mode of delivery observed in three smaller studies [Hino et al., 1998; Lin et al., 1998; Ohto et al., 2000].

6.1.3 OBJECTIVE 3: TO DETERMINE THE PREVALENCE AND CORRELATES OF GBV-C INFECTION AMONG HIV-INFECTED AND HIV-UNINFECTED PREGNANT WOMEN IN THAILAND

In a cross-sectional analysis, we observed that ever having GBV-C infection (i.e. presence of RNA or antibody) was more common among HIV-infected compared to HIV-uninfected pregnant women (33% and 15%, respectively). Prevalence of RNA was almost 3 times higher among HIV-infected women (20% compared to 7%) and prevalence of antibody was 1.6 times higher (13% compared to 8%) indicating an increased risk of GBV-C infection among HIV-infected women likely due to common risk behaviours, and possibly, a reduced ability to clear infection among HIV-infected women. In addition to HIV status, GBV-C infection was independently associated with more than one lifetime sexual partner and injection drug use confirming results of previous studies suggesting both parenteral and sexual modes of transmission. As crowding (as measured by the number of people in a household) was not associated with GBV-C infection in our study, transmission of GBV-C genotypes 2, 3, and 4, the genotypes observed in our study, through casual contact appears to be unlikely. As
multiple genotypes were observed among 7% of women in our study, infection with one genotype does not appear to prevent subsequent infection, or possibly simultaneous transmission, with a second genotype.

Although our study was cross-sectional and we did not directly observe GBV-C clearance, we estimated predictors of clearance by comparing prevalence of antibody alone to prevalence of RNA among women ever having had GBV-C infection. Clearance of GBV-C was less common among HIV-infected compared to HIV-uninfected persons, possibly due to slower clearance by HIV-infected women as previously observed [Devereux et al., 1998] or to a more rapid deterioration of antibody among HIV-infected persons [Devereux et al., 1998; Stark et al., 1999]. In addition, clearance of GBV-C was independently associated with older age (likely a proxy for duration of infection) as previously observed [Rey et al., 2000] and more than 10 lifetime sexual partners (possibly also related to duration of infection). Among HIV-infected women, clearance of GBV-C was more likely for women with a viral load of <10,000 viral copies/mL, a new finding.

Our large study contributes to the understanding of existing knowledge on risk factors for GBV-C infection and clearance in a unique population of HIV-infected pregnant women in Thailand, confirming parenteral and sexual risks of infection and reduced prevalence of antibody among HIV-infected persons. The association between high HIV viral load and a lower prevalence of GBV-C antibody is a new finding and is consistent with the decreased prevalence of GBV-C antibody observed among HIV-
infected compared to uninfected persons; a stronger immune system may be needed to clear GBV-C and to develop antibody to the GBV-C virus.

6.1.4 INTERACTION BETWEEN GBV-C AND HIV: CONTRIBUTION TO A CAUSAL MODEL

In addition to our stated objectives, our results also contributed to existing knowledge regarding the nature of the interaction between GBV-C and HIV (Figure 6.1) including: 1. an inverse association between MTCT of HIV and MTCT of GBV-C; 2. increased MTCT of GBV-C among women receiving ART; and 3. an association between clearance of GBV-C (with development and persistence of antibody) and HIV viral load <10,000 viral copies/mL. As HIV and GBV-C are likely acquired by an infant at similar times, it is not possible to tell which would be the causal antecedent variable. Other GBV-C studies have had similar dilemmas in interpreting their results. In two longitudinal studies, a beneficial effect of persistent GBV-C infection on survival disappeared with adjustment for time updated CD4+ count (itself strongly associated with, and part of the definition of, HIV progression) leading to opposite interpretations of the benefit of GBV-C (one interpreted GBV-C to be beneficial while the other did not) [Van der Bij et al., 2005; Williams et al., 2004]. HIV viral load has been inversely associated with GBV-C viral load. A recent study observed increased GBV-C replication and decreased HIV replication after receipt of HAART, suggesting that something related to HIV replication, possibly immune activation, may affect GBV-C replication [Bjorkman et al., 2007]; however, viral copy numbers reported were unusually low. Our study did not observe an association between GBV-C infection and HIV viral load or
CD4+ count, possibly because of the young age and early stage of HIV infection of our study population, as a beneficial effect of coinfection is thought to appear after several years of coinfection. Among GBV-C-HIV coinfected women, GBV-C and HIV viral load were correlated only among women receiving ART, although the scatter plot is not very conclusive (Figure 6.2).

Figure 6.1 Causal Diagram: contribution of our study

[Diagram showing causal relationships between maternal GBV-C viral load, maternal ART, infant GBV, and infant HIV viral load]
6.2 STRENGTHS AND LIMITATIONS

6.2.1 STRENGTHS

With data drawn from three separate perinatal studies from the same population at different points in time, we had a large sample size with which to examine study covariates. This is very important in studies of viral coinfection and viral interaction as well as studies of perinatal transmission, where the sample size for analysis is progressively reduced, first by the number of coinfected persons, and second, by the number of infected and coinfected infants. In addition, because each study stood on its own, we could examine the consistency of associations across studies, strengthening our findings.
In addition, we had access to good quality data, both questionnaire and laboratory, from these CDC-sponsored studies, with high quality control assured during the studies by double data entry, logical data checking programs and systematic error reports, a very rigorous laboratory, storage of our specimens at -80ºC, and testing of specimens at the CDC HIV/AIDS Retrovirology laboratory in Atlanta.

Because GBV-C genotypes 2, 3, and 4 were prevalent in our study (42%, 35%, and 16% respectively among HIV-infected women), and because we found no difference in associations examined by genotype, our findings are generalizable to populations in Europe, North America and Asia, but possibly not to populations in Africa or Indonesia where genotypes 1 & 5, and 6 have been observed.

Because few subjects were lost to follow-up, no potential biases are likely to have arisen from this loss. Blood specimens were available and tested for 97% of women enrolled in the original studies. Of 327 infants born to GBV-C RNA-positive and RNA-indeterminate mothers, we tested all but 40 (12%) infant specimens (Appendix 2). Of these 40, 11 were HIV-infected, 14 HIV-uninfected and 15 had HIV status unknown. Sensitivity analysis reveals that our main finding would still be of borderline significance assuming 41% MTCT of GBV-C and 44% MTCT of HIV (Appendix 4, TableA.4.3). The higher than observed estimate of MTCT of HIV of 44% was used in this analysis, to account for the possibility that missing infants may be more likely to be HIV-infected than the other infants in the study (MTCT of HIV 13%). However, given our findings that 6.5% of HIV-infected infants were GBV-C infected, it would be unlikely that 41% of
the missing HIV infected infants would be GBV-C infected. Given that the direction of effect is the same and that the association of infant GBV-C with MTCT of HIV remains of borderline significance under rather extreme assumptions, we conclude that loss-to follow-up would not have biased our study conclusions.

6.2.2 LIMITATIONS

6.2.2.1 Sensitivity of the GBV-C test

The lack of a gold standard test for GBV-C RNA is a problem common to all studies of GBV-C. While specificity was observed to be relatively good, sensitivity was problematic in the three quality control studies that we reviewed [Bogard et al., 1997; Kunkel et al., 1998; Lefrere et al., 2000a]. Where a gold standard does not exist it is important to use the best laboratory procedures available and to try to quantify the effect of potential misclassification. We used the laboratory of Dr. Salvatore Butera (Chief, Virology Section, HIV/AIDS and Retrovirology Branch, Division of AIDS, STD, and TB Laboratory Research, U.S. Centers for Disease Control and Prevention), where tests for detection and genotyping of GBV-C had been previously developed. We also conducted a validation study in the form of a specimen exchange with the University of Iowa, a laboratory experienced in GBV-C research (see Appendix 3). While the results of the validation study led us to conclude that sensitivity could be a problem for our study, a recent study by our validation study collaborators at the University of Iowa, observed that the 5’UTR primer, the one used for our study, was the most sensitive of 5 primers tested (100%) and that specificity was 90% in a nested-PCR test for GBV-C [Souza et al.,
2006]. They recommended using the 5’ UTR primer to screen for GBV-C and then another more specific primer to confirm presence of GBV-C. Nested PCR increases both the sensitivity of the assay by reamplifying the product of the first reaction in a second reaction and the specificity because the inner primers amplify only if the first PCR reaction yields a specific product. In our study we used genotype testing to confirm presence of GBV-C in maternal samples, enhancing the specificity of our results. While we used the single best primer available, we did not use nested PCR and sensitivity of our test may be less than 100%. Misclassification tends to bias associations to the null unless it is differential by exposure. Despite the potential for random misclassification which would have biased our results to the null, the observed inverse association between MTCT of GBV-C and MTCT of HIV was strong and, after sensitivity analysis, we concluded that our conclusions would not change even if an additional 100 infants had been misclassified as GBV-C negative. Of concern would be the possibility that presence of HIV somehow interferes with the test for GBV-C, making coinfection appear unlikely. However, as 20% of HIV-infected women tested GBV-C positive, this would not have been possible.

Several observations from our study add to our confidence regarding the sensitivity of our GBV-C test and the accuracy of our viral load measurement. The prevalence of GBV-C RNA was in the range observed in other studies of HIV-infected pregnant women, the rate of MTCT of GBV-C was similar to that observed in other studies of HIV-infected pregnant women, a dose-response relationship existed between maternal GBV-C viral load and MTCT of GBV-C similar to other vertically transmitted
viruses and reported risks for GBV-C infection such as lifetime number of sexual partners and age at first sex were associated in a dose-response manner with GBV-C infection. Our ability to replicate findings observed in previous studies also added to our confidence regarding GBV-C measurement in our study. We replicated the findings of two studies observing that maternal GBV-C infection does not reduce MTCT of HIV and the findings of three studies observing that mode of delivery and GBV-C viral load are associated with MTCT of GBV-C. There were no observations in our study that conflicted with results published in the MTCT of HIV or MTCT of GBV-C literature. The literature regarding the interaction between HIV and GBV-C contains many conflicting results. However, the weak inverse correlation we observed between GBV-C and HIV viral load among women receiving ART was similar to correlations observed in other studies.

Despite our general confidence in the GBV-C testing for our study, we observed differences between the studies in GBV-C prevalence and GBV-C viral load after controlling for other factors that we cannot explain and which we have investigated further. GBV-C infection was more prevalent but GBV-C viral loads were significantly lower among HIV-infected women in Peri-1 compared to Peri-2 and -3. The higher prevalence of GBV-C RNA among women in Peri-1 compared to the other two studies is curious given the lower viral load which would have made detecting GBV-C more difficult. However, many Peri-1 indeterminate specimens were confirmed with genotype testing (40 of 54 indeterminate specimens) compared to two of five in Peri-2 and 5 of 41 in Peri-3.
The median GBV-C viral load among coinfected women was significantly lower in Peri-1 compared to both Peri-2 and-3 combined. Possible explanations for viral load differences between studies include differences in length of storage of specimens, testing differences, and population differences. Peri-1 samples were stored in liquid nitrogen (-196°C) for 10 to 12 years which may have caused deterioration in levels of detectable virus, whereas Peri-2 and Peri-3 samples were stored in freezers at -80°C for periods of 0-8 years. As rates of MTCT-GBV were similar across all studies among women not receiving ART (Chapter 4, Table 3) and because maternal GBV-C viral load is a major determinant of MTCT-GBV-C, a similar level of virus may have existed in Peri-1 samples prior to freezing; however, we cannot be sure. The same methods and laboratory were used for all tests; however, the laboratory technician performing the quantitative RT PCR testing was different for Peri-1 than for Peri-2 and Peri-3. However, quality control for the RT-PCR test was assured by several means including a standard curve, negative controls, and human plasma standards of known quantities of virus ranging from \((10^2-10^6)\) which were extracted and run on each plate. Standards were changed once over the course of the study but were mixed by the same person. Standards in the lower range where the qualitative determination of GBV-C infection is made were not different between studies or batches and would not have affected our conclusions. While quantification of the \(10^6\) standard between batches appeared slightly higher in Peri-2 and-3 compared to Peri-1, the mean viral load difference was only \(0.2 \log_{10}\) copies and would not have explained the observed difference in viral load.
The genotype confirmation test of the RT-PCR positive specimens and the use of negative controls make it unlikely that the high prevalence of GBV-C observed in Peri-1 was due to laboratory error. Also, despite the lower viral loads observed, maternal GBV-C viral load was predictive of MTCT of GBV-C in a dose response relationship in Peri-1 as it was in Peri-2 and -3 and, a test of homogeneity did not reveal any significant differences. The lower GBV-C viral loads observed in Peri-1 may be due to prolonged storage of specimens and, while reasons for the higher prevalence of GBV-C are unknown, it is possible that population differences (perhaps stage of epidemic, length of infectivity) may be responsible.

Combining three separate studies can be problematic in some cases. However, we do not believe that heterogeneity was an issue for our study. Firstly, the study populations, all pregnant women attending Rajavithi or Siriraj hospitals for prenatal care and delivery, were members of the same population at different points in time. The main difference between the studies was the availability of ART. If the first study had lasted 12 years, then women from all three studies would have been in the same study, with a variable to adjust for potential confounding by ART. We adjusted all analyses for potential confounding by ART and other unmeasured study differences. We also examined potential effect modification by ART on the effect of maternal GBV-C on MTCT of HIV by adding an interaction term between maternal GBV-C and ART to the logistic regression model; it was not significant. No statistically significant associations between maternal GBV-C and MTCT of HIV were observed in any of the three studies and, the confidence limits of the estimates in all three studies overlapped (p 92, Table 3).
We assessed the association of every variable with each outcome across the three studies with the exception of ART which was assessed across two studies as ART was not available to women in Peri-1. We could not reject the null hypothesis of homogeneity for any of the associations. However, because other unmeasured potential confounders may have changed over time, possibly affecting the baseline risk of the outcome within each study, we added a study variable to control for differences in baseline risk between studies. This may not have been necessary if all potential confounders were included in our model; however, the downside of including the study variable is a loss of statistical efficiency versus potentially biased estimates if we failed to adjust for baseline study differences. We feel the potential benefits outweigh the potential disadvantages.

6.2.2.2 Study Design Issues

6.2.2.2.1 Infants of GBV-C RNA-negative women were not tested for GBV-C infection and were assumed to be GBV-C negative

Ideally, a sub-sample of infants of GBV-C RNA-negative women would have been tested for GBV-C RNA. However, we believe that most infants of GBV-C RNA-negative mothers would have tested GBV-C RNA negative. In three studies conducted in Europe, all infants of GBV-C RNA-negative mothers tested GBV-C RNA negative [Wejstal et al., 1999] [Lefrere et al., 2000b] [Barqasho et al., 2004]. In our study, no infants of 38 GBV-C RNA-indeterminate mothers (from 1 to 999 viral copies and no genotype detected) tested positive for GBV-C RNA. However, two African studies have
reported a small number of GBV-C RNA-positive infants born to apparently GBV-C RNA-negative mothers implying either horizontal transmission of GBV-C, poor sensitivity of the maternal GBV-C test, or laboratory contamination of the infant test. As discussed in Chapter 3, the balance of evidence supports vertical transmission of GBV-C, especially in early infancy, and we feel that horizontal transmission is unlikely to have occurred in our study. However, even if 100 infants were misclassified as negative and 13 (13%) of these infants were HIV-infected, the observed association between MTCT of HIV and MTCT of GBV-C would still be statistically significant as previously discussed in Chapter 3. In addition, the observed association between MTCT of HIV and MTCT of GBV-C using only tested infants of GBV-C RNA mothers, stands alone.

6.2.2.2.2 No genotype confirmation for infants with low GBV-C viral loads
   Because initially GBV-C genotype testing was performed to determine genotype and not to confirm the PCR test result, we felt it reasonable to assume that mothers and infants had the same GBV-C genotype. As only 7 (2.4%) of 287 infant specimens were classified as indeterminate, the lack of a genotype confirmation would not have substantially affected our results. One of the GBV-C indeterminate specimens was HIV-infected and six were HIV-uninfected and, if we assume that all were GBV-C-infected or GBV-C-uninfected, this would not change our main finding.

6.2.2.2.3 Different results between plasma and serum
   We compared GBV-C viral load between HIV-infected (plasma specimens) and HIV-uninfected (serum specimens) women in Peri-1 (Appendix 4, Table A4.2), although for our study, the qualitative determination of GBV-C status was the focus of our study. Available specimens for measuring GBV-C viral load included plasma specimens for
HIV-infected women and serum specimens for HIV-uninfected women; ideally, all measures would have been made using the same type of specimen. We hypothesized that serum specimens could potentially result in lower viral load detection as clot formation in serum may trap viral particles. However, we found that while GBV-C was less prevalent in Peri-1 HIV-uninfected women (7% vs. 32%) probably due to differences in risk behaviours, GBV-C viral loads were significantly higher in HIV-negative women, contrary to our expectation. This might suggest that GBV-C is better able to proliferate in the healthier immune environment of HIV-uninfected individuals, or perhaps that something related to plasma specimens, heparin, for example, might reduce viral load or viral load detection. As all HIV-infected samples were plasma and all HIV-uninfected samples were serum, it is not possible to separate these effects (i.e. perfect confounding). Based on the prevalence of GBV-C observed, which were in the range expected for these populations, we have no reason to suspect that the qualitative determination of GBV-C status was affected by differences in blood specimens.

6.2.2.2.4 Problems with cross-sectional data: incidence/prevalence biases (duration ratio bias and point prevalence complement bias), and unobserved clearance

Baseline cross-sectional data collected at enrollment were analyzed to determine correlates of ever having GBV-C infection. A cross-sectional study yields prevalence rather than incidence estimates, and the use of prevalence data to estimate causal associations may bias some estimates. Prevalence rate ratios (PRR) tend to underestimate the underlying incidence rate ratio (IRR) due to a ‘point prevalence complement ratio bias’ as described below [Szklo and Nieto, 2000].
The point prevalence odds = \( \frac{\text{point prevalence}}{1 - \text{point prevalence}} \times \frac{\text{incidence} \times \text{duration}}{\text{ incidence} \times \text{duration} \times (1 - \text{ point prevalence})} \)

therefore,

\[ \text{point prevalence} = \text{ incidence} \times \text{ duration} \times (1 - \text{ point prevalence}) \]

and,

\[ \text{PRR} = \frac{\text{incidence} \times \text{duration} \times (1 - \text{point prevalence})_{\text{exposed}}}{\text{incidence} \times \text{duration} \times (1 - \text{point prevalence})_{\text{unexposed}}} \]

The point prevalent complement ratio is \( \frac{(1 - \text{point prevalence})_{\text{exposed}}}{(1 - \text{point prevalence})_{\text{unexposed}}} \).

[Szklo and Nieto, 2000].

If prevalence of GBV-C infection was 33% among HIV-infected women and 15% among HIV-uninfected women, the point prevalence complement ratio would be 0.67/0.85 = 0.78, and the PRR would underestimate the IRR by 22%.

In addition, as prevalence is affected by the duration of the disease and, if duration of disease is differential with respect to exposure, then the prevalence rate ratio is biased by the duration-ratio bias, a form of survival bias [Szklo and Nieto, 2000]. However, if HIV-infected women cleared GBV-C RNA more slowly but lost antibody more rapidly, these two effects might counterbalance each other with respect to associations with ever being GBV-C infected. Another potential survival bias could occur if women with GBV-C infection survived longer than women without GBV-C infection among HIV-infected women but not HIV-uninfected women, making GBV-C
infection appear more common among HIV-infected women. However, a survival bias is unlikely given the relatively young age and early stage of HIV-infection of women in our study.

Because this study was not longitudinal, clearance was not directly observed but rather inferred from antibody presence. If women cleared RNA but either did not develop antibody or lost antibody before our blood sample was taken, this would result in misclassification. If HIV-infected women lost antibody more rapidly than HIV-uninfected women, then differential misclassification could bias the association between HIV status and clearance, making it appear as though HIV-infected women cleared virus more slowly.

6.3 IMPLICATIONS FOR FUTURE RESEARCH

GBV-C, a virus not known to cause any disease, is not by itself of critical public health importance. The main importance of GBV-C infection remains the nature of its interaction with HIV and potential for a beneficial effect on HIV progression and possibly, as observed in our study, MTCT of HIV. There is not yet a unifying theory of GBV-C-HIV interaction. There is still disagreement on the effects of GBV-C and much further research is required to sort out the biological mechanisms and pathways involved in the interaction between the two viruses. If an interaction occurs at the level of complex immunologic responses, in particular cytokine responses, where there are many loops and feedback mechanisms, the pathways are not straightforward. Many different
immunologic processes interact with each other and further research will be required to completely understand the mechanisms at work. The temporality of the interaction is also problematic for sorting out the causative factors, as the interaction between GBV-C and HIV seems to occur very rapidly.

The inverse association between MTCT of HIV and MTCT of GBV-C we observed is intriguing and may provide new information regarding a mechanism for the prevention of MTCT of HIV. The results of our study need to be confirmed by other studies and the underlying causal pathway and mechanisms need to be identified. For example, infant GBV-C infection may influence the cytokine milieu in such a way as to reduce the risk of infant HIV acquisition and cytokine profiles of infants with and without GBV-C infection could be examined. It would also be interesting to investigate whether or not GBV-C infection affects the placenta in such a way as to promote GBV-C transmission but inhibit HIV transmission (both chorioamnionitis and placental malaria have been associated with reduced MTCT of HIV in some but not all studies) [Ayisi et al., 2004; Naniche et al., 2008; Schwartz et al., 2000]. Frequent serial measures of GBV-C and HIV viral detection in the early newborn period would be of use in order to try to observe any potential early interaction between the two viruses in the newborn, but may be difficult for several reasons. Firstly, it may be difficult to obtain consent for frequent infant blood draws, infant specimens can be difficult to obtain, and the volume of blood obtained can be low. Also, as HIV can be detected at 50 copies/mL or more while GBV is generally detected at much higher viral loads (50,000 copies/mL in our study), time of detection may not equal time of infection. As there appear to be very few coinfected infants by 4-6
months, serial measures of GBV-C and HIV viral load in coinfectected infants may have small sample sizes but would still be useful. In future studies, I would recommend testing at least a sub-sample of infants of GBV-C RNA-negative mothers and also genotyping GBV-C RNA-positive infants to confirm infection from the mother.

Our study of MTCT of GBV-C contributed the first large study to the body of knowledge of MTCT of GBV-C and the observed association between ART and increased MTCT of GBV-C adds another piece to the interesting puzzle of the interaction between HIV and GBV-C. Further investigations to sort out the biologic pathway for this association as well as the inverse association between MTCT of GBV-C and MTCT of HIV would be useful. If GBV-C infection becomes important in and of itself, or because of its interaction with HIV infection, more large studies would be required to examine predictors of MTCT of GBV-C.

Correlates of GBV-C infection appear to be well known. However, clearance of GBV-C is less well characterized and future research in this area may add important insights into the nature of the interaction between HIV and GBV-C. In the context of GBV-C and HIV coinfection, it would be useful to examine GBV-C clearance with frequent serial measurements to determine if HIV infected persons are less likely to clear GBV-C or to develop and maintain antibodies, and to determine the implications of different clearance patterns for HIV progression and survival. Previous studies found that those who lost GBV-C (most without detectable antibodies) had the worst survival
and the biological mechanism for this remains unknown. The role of CD4+ count and HIV viral load in this context could be examined.

Additional validation studies of the RT GBV-C PCR testing would also be helpful. A common panel for testing GBV-C would be preferable to a specimen exchange, as it is difficult to interpret discordant results when there are only two laboratories. The development of more sensitive quantitative GBV-C RT-PCR tests would also be useful, especially for infant testing in the very early stages of infection, when viral loads may be quite low.

In conclusion, GBV-C infection in women was associated with both sexual and parenteral risk behaviours. Antiretroviral therapy appeared to increase MTCT of GBV-C in our study, a finding that needs to be further explored. Contrary to our initial hypothesis, MTCT of HIV was not reduced with maternal GBV-C infection. However more importantly, we identified for the first time, that infant GBV-C acquisition was associated with a reduced rate of MTCT of HIV. Uncovering the mechanisms involved may lead to possible interventions for the prevention of transmission of HIV.
6.4 REFERENCES


APPENDICES

Appendix 1: Author’s contribution to the study

Appendix 2: Population trees with study results

Appendix 3: GBV-C Viral Detection Validation Study

Appendix 4: Additional analyses
Appendix 1

AUTHOR’S CONTRIBUTION TO THE STUDY

In accordance with the recommendations of the committee that examined the protocol for this thesis, the following information outlines the specific contribution of the author to both the original perinatal studies and the thesis.

THE BANGKOK SHORT-COURSE ZIDOVUDINE TRIAL (1996-98)

The author was hired as an epidemiologist with the HIV/AIDS Collaboration, a collaboration between the Thailand Ministry of Public Health and the U.S. Centers for Disease Control and Prevention, to assist with the Bangkok short-course zidovudine trial, one of three perinatal studies whose data we used for the current study. The author was responsible for standard operating procedures and protocol adherence, hospital and laboratory coordination, quality control and error checking, and adverse event reporting and, also participated in questionnaire development. The author also analyzed data for a placenta substudy from the first perinatal study [Schwartz et al., 2000].

The author was responsible for all aspects of the GBV-C/MTCT of HIV study including study design, protocol development, and grant submissions. Funding was received from both the NIH and CIHR to conduct this study. Ethical submissions were required in Canada, the U.S. and Thailand and some were obtained with assistance from collaborators at the CDC and in Thailand. Three collaborator meetings were held in Bangkok to consult with Thai colleagues regarding the use of specimens, study protocol, and preliminary analyses (April 2003, July 2004, and December 2006). The author was responsible for arranging sample shipments from Bangkok to Atlanta for GBV-C testing, for ordering laboratory supplies, and for checking and consulting regarding laboratory quality control, procedures and decisions. The author also made a site visit to the laboratory in Atlanta to oversee laboratory procedures and testing protocols. The data base consisting of the original study and laboratory data bases and the GBV-C laboratory data was assembled by the author, and data was checked for accuracy and duplicates. Data analysis was conducted by the author and findings have been presented at national and international conferences. The author has also written three manuscripts, one of which has been published in the Journal of Infectious Diseases, one of which is in press at the Journal of Infectious Diseases, and the third has been submitted to the CDC for approval for submission to the Journal of Medical Virology.

Appendix 2
Population trees with study results
Peri-1 mothers

**Peri-1 Maternal GBV-C Status**

- **342 HIV-infected pregnant women**
  - GBV-C RNA RT-PCR
    - 93 GBV-C RNA+ (33 EIA +, 5 Anti-E2 +)
    - 187 GBV-C RNA- (33 EIA -, 154 Anti-E2 -)
    - 14 GBV-C RNA indeterminate

- **344 HIV-uninfected pregnant women**
  - GBV-C RNA RT-PCR
    - 294 maternal delivery specimens
    - 93 GBV-C RNA+ (33 EIA +, 5 Anti-E2 +)
    - 187 GBV-C RNA- (33 EIA -, 154 Anti-E2 -)
    - 11 GBV-C RNA indeterminate

- **Peri-1 infants**

**Peri-1 Infant GBV-C Status**

- **Maternal GBV-C status Of HIV-infected mothers**
  - 8 GBV-C RNA negative
  - 2 GBV-C RNA indeterminate
  - **GBV-C RT-PCR**
    - 10 infant specimens tested
      - 4 no specimen
        - 1 stillborn, 3 other

- **Infant GBV-C status**
  - 154 assumed negative
  - **GBV-C RT-PCR**
    - 10 infant specimens tested
      - 4 no specimen
        - 1 lost, 3 other

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*40/54 (74%) with low viral load genotyped and were classified as GBV-C RNA+

** 8/19 with low viral load genotyped and were classified as GBV-C RNA+
Peri-2 mothers

Peri-2 Maternal GBV-C status

GBV-C RNA RT-PCR
397 maternal delivery specimens

62 GBV-C RNA+ 332 GBV-C RNA-

EIA

1 Anti-E2+ 61 Anti-E2-

62 GBV-C RNA+ 47 GBV-C antibody positive 285 GBV-C negative 3 GBV-C RNA indeterminate

EIA

47 Anti-E2+ 285 Anti-E2-

62 GBV-C RNA+ 47 GBV-C antibody positive 285 GBV-C negative 3 GBV-C RNA indeterminate

EIA

3 GBV-C RNA Indeterminate*

*2/5 (40%) with low viral load genotyped and were classified as GBV-C RNA positive

Peri-2 infants

Peri-2 Infant GBV-C Status

Maternal GBV-C status of HIV-infected mothers

62 GBV-C RNA positive 47 GBV-C antibody positive 285 GBV-C negative 3 GBV-C RNA indeterminate

Infant GBV-C status

6 no specimen (4 moved, 2 other)

GBV-C RNA RT-PCR
56 infant specimens tested

29 GBV-C RNA positive 285 GBV-C RNA negative

47 assumed negative

2 GBV-C RNA indeterminate 285 assumed negative

GBV-C RNA RT-PCR
2 infant specimens tested

29 GBV-C RNA positive 285 GBV-C RNA negative

2 GBV-C RNA indeterminate 285 GBV-C RNA negative

0 GBV-C RNA positive 2 GBV-C RNA negative

0 GBV-C RNA indeterminate
Peri-3 mothers

**Peri-3 Maternal GBV-C status**

759 women enrolled by March 31, 2004

10 did not consent storage of blood

GBV-C RNA RT-PCR
749 maternal delivery specimens

- 119 GBV-C RNA positive
- 594 GBV-C RNA negative
- 36 GBV-C RNA indeterminate*

*5/41 (12.2%) with low viral load genotyped and were considered GBV-C RNA positive

Peri-3 infants

**Peri-3 Infant GBV-C Status**

Maternal GBV-C status of HIV-infected mothers

- 119 GBV-C RNA positive
- 105 GBV-C antibody positive
- 489 GBV-C negative
- 36 GBV-C unknown

Infant GBV-C status

- 110 infant specimens tested
- 105 assumed negative
- 489 assumed negative
- 26 infant specimens tested

GBV-C RNA RT-PCR
51 GBV-C RNA positive
59 GBV-C RNA negative
5 GBV-C RNA indeterminate
105 GBV-C RNA negative
489 GB-C RNA negative
5 GBV-C RNA positive
26 GBV-C RNA negative
1 GBV-C RNA indeterminate
All mothers

Maternal GBV-C Status (all studies)

1783 maternal specimens from 3 perinatal studies in Thailand

GBV-C RNA RT-PCR
1440 HIV-infected maternal specimens

274 GBV-C RNA+
1113 GBV-C RNA-
53 GBV-C RNA indeterminate* 

EIA

12 Anti-E2 +
50 Anti-E2-
185 Anti-E2 +
262 Anti-E2-
928 GBV-C antibody positive
11 GBV-C RNA indeterminate
24 GBV-C RNA positive

GBV-C RNA RT-PCR
343 HIV-uninfected maternal specimens

274 GBV-C RNA+
1113 GBV-C RNA-
53 GBV-C RNA indeterminate* 

EIA

1113 Anti-E2 +
53 Anti-E2-
42 Anti-E2-
11 GBV-C RNA indeterminate

24 GBV-C RNA positive

*47/100 with low viral load genotyped and were classified as GBV-C RNA positive (most Peri-1)
All infants

**Infant GBV-C Status (all studies)**

- **Maternal GBV-C status (HIV-infected mothers only)**
  - 274 GBV-C RNA positive
  - 185 anti-E2 positive
  - 928 GBV-C negative
  - 53 GBV-C unknown

- Infant GBV-C status
  - GBV-C RNA RT-PCR
    - 249 infant specimens tested
      - 185 assumed negative
      - 928 assumed negative
  - 25 no infant specimen

- GBV-C RNA RT-PCR
  - 38 infant specimens tested
    - 35 GBV-C RNA positive
    - 3 GBV-C RNA indeterminate

- 274 GBV-C RNA positive
- 144 GBV-C RNA negative
- 4 GBV-C RNA indeterminate
- 185 GBV-C RNA negative
- 928 GBV-C RNA negative
- 0 GBV-C RNA positive
- 53 GBV-C RNA unknown

Total infants:
- 101 GBV-C RNA-positive
- 1,292 GBV-C RNA negative
- 7 GBV-C RNA indeterminate
APPENDIX 3

GBV-C VIRAL DETECTION VALIDATION STUDY

Characterizing qualitative agreement of GBV-C RT-PCR performed at the HIV/AIDS and Retrovirology Branch, U.S. Centers for Disease Control and Prevention, Atlanta and the University of Iowa, Iowa City (2004)

3.1 BACKGROUND AND RATIONALE

At present, there is no gold standard to detect GBV-C virus by RT-PCR. RT-PCR for GBV-C is still considered experimental and each laboratory develops its own methods, including preparation of primers for amplification of GBV-C viral RNA. Different primers amplify different portions of the GBV-C genome and discordant results may be obtained with different primers. The reliability of PCR testing for GBV-C is therefore of concern.

Three quality control studies found that the sensitivity of GBV-C PCR testing can be problematic [Bogard et al., 1997; Kunkel et al., 1998; Lefrere et al., 2000]. A study from Germany using an established reference panel of 10 specimens in 14 laboratories found that, although results were similar between laboratories regardless of RNA extraction and PCR methods (in-house, commercial kit, single-round, nested, probe hybridization), the type and number of genomic regions (5'-noncoding, NS3, NS5), or the isolate type (genotype), 29% (4) reported perfect results, 29% (4) reported false positive
results and 64% (9) reported false negative results in dilutions above $10^3$ compared to the reference panel. Dilutions of $10^2$ - $10^3$ genome equivalents/mL were not counted as false negative as the developers of the panel also had difficulty detecting them [Kunkel et al., 1998]. In a study of 20 specimens tested at 22 laboratories in France, specificity was excellent (100%) in 20/22 laboratories while sensitivity was acceptable (85% to 100%) in 15 centres and inadequate (38% to 77%) in seven centres [Bogard et al., 1997]. Another study from France tested a panel of 20 serum samples using a two-stage methodology and found a surprising heterogeneity of results among 18 laboratories using a commercialized (but not standardized) assay [Lefrere et al., 2000]. Sensitivity varied from 23% to 92% and was 82% overall in the second stage of the study. Specificity was good or excellent in all studies but a few false positives were thought to have been caused by a carryover of already amplified RNA [Kunkel et al., 1998].

These studies identified the following measures to improve the quality of laboratory results: 1) participation in repeated quality control studies; 2) evaluation of the sensitivity and specificity of each laboratory prior to publication of epidemiologic studies that rely on viral detection of the GBV-C genome by PCR assays [Lefrere et al., 2000] [Bogard et al., 1997]; and 3) use of internal and external standards and efforts to prevent DNA contamination [Kunkel et al., 1998].

In the context of our study ‘The impact of GBV-C coinfection on perinatal HIV transmission’, we attempted to address reliability concerns by exchanging specimens between the HIV/AIDS and Retrovirology Laboratory, U.S. Centers for Disease Control
and Prevention (CDC) and the University of Iowa (UOI) as suggested by one of our funding agencies, the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Our quality control procedures also included both standard curves, use of human plasma standards and negative controls to validate both the extraction and amplification stage of PCR testing. It was important that our study accurately identify and classify GBV-C viremic subjects. Poor sensitivity of the GBV-C PCR test could result in differential or non-differential misclassification and bias the true measure of the effect of GBV-C infection on mother-to-child HIV transmission, the main objective of the study.

3.2 METHODS

3.2.1 SPECIMEN SELECTION

3.2.1.1 CDC specimens

CDC sent 100 specimens to UOI, including 90 extracted RNA specimens and 10 plasma standards (three negative standards and seven positive standards) in dilutions varying from $10^2$ to $10^6$, including duplicates of the $10^5$ and $10^6$ standards. UOI was able to analyze 99 of the 100 specimens received from CDC; one did not have a sufficient quantity. We are therefore able to compare 99 matching results.
3.2.1.2 UOI specimens

UOI sent two shipments of plasma specimens to CDC. CDC received 52 specimens in the first (100 sent, 48 were duplicates, and were recalled) labeled 1 through 52; and 100 in the second (98 with complete ID numbers). The total number of UOI specimens tested at CDC was 150.

A specimen list sent to CDC from UOI indicated 162 specimens, 81 specimens in the first shipment, 71 in the second shipment and 10 duplicates. Another list was later received for the first 52 specimens with lab numbers 1 through 52. Seventeen of the first 52 specimens received by CDC were not on the UOI specimen list. The result from one UOI specimen remained unconfirmed. Overall, UOI results were received from UOI for 169 specimens, 10 of which were duplicates.

With 159 unique results from UOI and 150 results from CDC, we were able to match the results for 145 specimens (20 results were not matched; 5 specimens sent to CDC did not have a UOI result and 15 results from UOI had no identifiable specimen for CDC to test).

UOI’s specimen list indicated that 10 duplicates had been sent. However, only one duplicate was received by CDC (one tube labelled #4 in the first shipment and micro ID# 2316079 in the second shipment).
3.2.2 PREPARATION AND TREATMENT OF SPECIMENS

3.2.2.1 CDC specimens sent to UOI

CDC specimens sent to UOI were tested using the methods described in Section 2.3 below. The plasma standards were quantified with repeat testing using real-time RT-PCR and show a variability of less than 0.5 log copies. These standards are routinely used as controls in the real time RT-PCR assay and have shown good reliability; standards over $10^2$ copies consistently amplify. GBV-C positive specimens sent to UOI were confirmed with genotyping.

3.2.2.2 UOI specimens sent to CDC

UOI specimens came from the UOI HIV/AIDS clinic. GBV-C RNA testing was performed as described below. RT-PCR was performed on RNA extracted from plasma using primers from five different GBV-C genome regions.

Specimens were marked with a unique identifier and were analyzed blind to previous designation by laboratory personnel.

3.2.3 LABORATORY METHODS
3.2.3.1 CDC: Methods and primers

**RNA Extraction** – GBV-C viral RNA was extracted from 200 uL of plasma using the QIAamp MinElute Virus Vacuum kit (Qiagen Inc., Valencia, Calif.), according to the manufacturer’s instructions. The volume of the final eluate was 50 uL.

**Quantitative RT-PCR assay for determination of GBV-C viral load:** Five microliters of RNA extracted as described above were used in a quantitative real-time RT-PCR assay based on TaqMan™ technology using the Quantitect Probe RT-PCR kit (Qiagen Inc., Valencia, Calif.) according to the manufacturer’s instructions. The reaction volume was 50 uL and the number of reaction cycles was 40. The primers used amplify a 105-base pair fragment in the conserved 5' untranslated region (UTR) of GBV-C. The sequences of the primers and the dual-labeled probe are as follows (all nucleotide numbers below refer to the sequence with accession number U44402 [Linnen et al., 1996].

Forward primer: GBV-C 03.1-F - 5' GCA CGG TCC ACA GGT GTT 3' (18-mer encompassing nucleotides 226-243).

Reverse primer: GBV-C 03.2-R - 5' GTA CGT GGG CGT CGT TTG 3' (18-mer encompassing nucleotides 313-330).

Probe: G-THAI-UTR-P-3 - 5' CCG ACG TCA GGC TCG TCG TTA AAC 3' (24-mer encompassing nucleotides 268-291). The probe is synthesized with the dye FAM on the 5' end and a dark quencher on the 3' end.
3.2.3.2 UOI: Methods and primers

**RNA Extraction** – GBV-C viral RNA was extracted from 140 uL of plasma using the QIAamp viral RNA minikit (Qiagen Inc., Valencia, Calif.) according to the manufacturer’s instructions. The volume of the final eluate was 80 uL.

**Qualitative RT-PCR assay for detection of GBV-C viral load:** RNA representing 25 uL plasma, extracted as described above, was used in a nested RT-PCR assay based as previously described [Xiang et al., 1998]. Primers were designed that amplify five separate regions of the GBV-C genome, including the conserved 5' nontranslated region (NTR), two regions in the E2 coding region, and conserved sequences in the NS3 and NS5A coding regions of GBV-C. The sequences of the primers and nucleotide numbers (based on GenBank AF121950) are shown below. Samples that had discordant results between primer sets were repeated and another sample within six months of the date on the same patient was also examined. To be called positive on the single sample, all five primer sets had to be positive. If there were any primer sets that did not amplify product, then positive was defined as having at least two genome regions test positive and that the same results were obtained when a different sample obtained from the same patient (obtained within 6 months of the initial sample date).

**E2 OUT:**

Sense: 5’ (1503) GDC GYG AYT CGA ARA TMG AYG 3’ (1523) (~ 772 bp)

AS: (2274) AAG ATC AAC GGG ACC AGC CGT GCC TCA (2248)
E2 NEST:
Sense: (1508) GAT ATC GAA RAT MGA YGT GTG GAG (1532)(~ 750 bp)
AS: (2262) TTA GGT ACC GCC TCA GCC AGC TTC AT (2237)

5’NTR OUT:
Sense: 5’ (59) AAG CCC CAG AAA CCG ACG CC3’ (~300 bp)
Anti-sense: (362) TGA AGG GCG ACG TGG ACC GT

5’NTR NEST:
Sense: 5’ (119) CGG CCA AAA GGT GGT GGA TG 3’ (~200 bp)
Anti-sense: (322) GTA ACG GGC TCG GTT TAA CG

NS5A OUT
Sense: 5’ (6668) ATG GTY TAY GGY CCT GGV CAA A 3’ (~245 bp)
Anti-sense: (6913) TAC TGC ART CYT CCA TGA TGA CAT

NS5A NEST
Sense: 5’ (6681) CTG GVC AAA GYG TYA CCA TT 3’ (~200 bp)
Anti-sense: (6883) TTC AAG AAT CCT CGC AGC ATT CT (6861)

NS3 OUT
Sense: 5’ (4558 ) GGT RWC CCT TGA TCC CAC CAT 3’ (~400 bp)
Anti-sense: 5’ (4953 ) CAC ATB GTC CGC TGA AC (4938) 3’

NS3 NEST
Sense: 5’ (4607) TCG GCW GAA YTG TCG ATG CA (4626) 3’ (~310 bp)
Anti-sense: (4919) ACG CCG CGH ACY TTT GCC CA (4891)

NE2 OUT
Sense: 5’ (~1455) TGT GGG GTT CCG TDT CTT GGT T (~1470) 3’
Antisense: 5’ (1910) RAA CGT HCC RCT VGG AGG CT (1931) 3’
NE2 NEST
Sense: 5’ (~1532) TGG NTC WGC CAG CTG YAC CAT AGC (~1555) 3’
Antisense 5’ (~1855) DTC YCG GAT CTT GGT CAT GG (1872) 3’

3.2.4 STATISTICAL ANALYSIS

Statistical analysis was performed using SAS version 8.2. Agreement was measured with a kappa statistic which measures the proportion agreeing over and above that expected by chance. Sensitivity and specificity were also calculated.

3.3 RESULTS

Results are presented first separately for each laboratory and then combined.
**3.3.1 CDC SPECIMENS**

Ninety-nine CDC specimens were tested by CDC and UOI. The test results showed ‘good’ agreement between laboratories with a kappa score of 0.74 [Altman, 1991]. Concordance was 88% (87/99) with 55 concordant negatives and 32 concordant positives. Of concern are the UOI test results of CDC’s 10 duplicates and standards (see below).

<table>
<thead>
<tr>
<th>Gold Standard:</th>
<th>UOI</th>
<th>CDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity of other lab:</td>
<td>82% (32/39)</td>
<td>86% (32/37)</td>
</tr>
<tr>
<td>Specificity of other lab:</td>
<td>92% (55/60)</td>
<td>89% (55/62)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>CDC Specimens</strong></th>
<th><strong>UOI results</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CDC results</strong></td>
<td><strong>Neg</strong></td>
</tr>
<tr>
<td>Neg</td>
<td>55</td>
</tr>
<tr>
<td>Pos</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
</tr>
</tbody>
</table>

McNemars test (p = 0.56)
Duplicates and standards:

Ten plasma standards, some in duplicate, were included in the 99 CDC specimens tested by UOI. The UOI results are listed below and show that 2 of 3 negatives were correctly identified and 4 of 6 positives were correctly identified for a 66% concordance (an additional incorrectly identified specimen, a $10^2$ dilution of the standard and below the limit of sensitivity for RT-PCR testing, is not included as a false negative in this analysis). The UOI laboratory found one high titer sample negative (#93), and upon further testing of this sample, it was negative using E2 and NS5A region primers, but positive using NS3 and 5’NTR primers. Because a second sample was not available for testing (within 6 months, as was done for the UOI samples), this was considered negative, despite 2 of 5 primers testing positive.

<table>
<thead>
<tr>
<th>CDC standards</th>
<th>UOI result</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^6$ standard (samples 93, 100)</td>
<td>N, P</td>
</tr>
<tr>
<td>$10^5$ standards (samples 96, 99)</td>
<td>P, P</td>
</tr>
<tr>
<td>$10^4$ standard (sample 97)</td>
<td>N</td>
</tr>
<tr>
<td>$10^3$ standard (sample 91)</td>
<td>P</td>
</tr>
<tr>
<td>$10^2$ standard (sample 94)</td>
<td>N (not counted as false negative)</td>
</tr>
<tr>
<td>Neg standards (samples 92, 95, 98)</td>
<td>N, P, N</td>
</tr>
</tbody>
</table>
Discordant results: No additional information is available for the 7 specimens identified as positive by UOI and negative by CDC. The following information is available for the 5 specimens that CDC identified as positive and UOI identified as negative:

5 Discordant Samples: CDC positive, UOI negative

<table>
<thead>
<tr>
<th>Sample</th>
<th>GBV copy number/reaction (20uL plasma)</th>
<th>GBV genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>76</td>
<td>4,750</td>
<td>No amplification</td>
</tr>
<tr>
<td>77</td>
<td>1,000</td>
<td>2A</td>
</tr>
<tr>
<td>94</td>
<td>10^2 standard</td>
<td></td>
</tr>
<tr>
<td>97</td>
<td>10^4 standard</td>
<td></td>
</tr>
<tr>
<td>93</td>
<td>10^6 standard</td>
<td></td>
</tr>
</tbody>
</table>

3.3.2 UOI SPECIMENS

145 UOI specimens were tested at both UOI and CDC laboratories. Overall, concordance was 71% (103/145) with 51 concordant negative and 52 concordant positive
specimens. The kappa score of 0.43 indicates low-moderate agreement [Altman, 1991].

UOI found more positive specimens than did CDC.

<table>
<thead>
<tr>
<th>Gold Standard:</th>
<th>UOI</th>
<th>CDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity of other lab:</td>
<td>62% (52/83)</td>
<td>83% (52/63)</td>
</tr>
<tr>
<td>Specificity of other lab:</td>
<td>82% (51/62)</td>
<td>62% (51/82)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test results</th>
<th>UOI results</th>
<th>CDC results</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC results</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>51</td>
<td>31</td>
</tr>
<tr>
<td>Pos</td>
<td>11</td>
<td>52</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>83</td>
</tr>
</tbody>
</table>

McNemars test (p = 0.002)

Duplicates:

One duplicate specimen was received by CDC; this specimen was identified as positive by UOI and negative by CDC on both tests.

Discordant results: The 31 specimens identified as positive by UOI and negative by CDC underwent further testing as discussed in Section 4 below. For the 11 specimens identified as positive by CDC and negative by UOI, seven had viral loads of
less than 1000 copies/reaction (the lower limit of reliable detection); the majority were from the first shipment (please see table below).

### 11 Discordant Specimens (CDC positive – UOI negative)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Shipment</th>
<th>GBV copy number/reaction (20uL plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>1</td>
<td>61</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>82</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>92</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
<td>110</td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>240</td>
</tr>
<tr>
<td>44</td>
<td>1</td>
<td>5,200</td>
</tr>
<tr>
<td>39</td>
<td>1</td>
<td>14,000</td>
</tr>
<tr>
<td>33</td>
<td>1</td>
<td>6,000,000</td>
</tr>
<tr>
<td>22891254</td>
<td>2</td>
<td>62</td>
</tr>
<tr>
<td>23241249</td>
<td>2</td>
<td>67</td>
</tr>
<tr>
<td>22962056</td>
<td>2</td>
<td>29,392</td>
</tr>
</tbody>
</table>

### 3.3.3 COMBINED DATA (CDC AND UOI SPECIMENS)

Combined, 244 specimens were available for analysis. There were 106 concordant negatives and 84 concordant positives for an overall concordance of 78% (190/244). The kappa score of 0.56 indicates 56% or moderate agreement over chance.
Gold Standard: UOI CDC

Sensitivity of other lab: 69% (84/122) 84% (84/100)

Specificity of other lab: 87% (106/122) 74% (106/144)

<table>
<thead>
<tr>
<th>UOI and CDC Specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>CDC results</strong></td>
</tr>
<tr>
<td><strong>UOI results</strong></td>
</tr>
<tr>
<td><strong>Neg</strong>    <strong>Pos</strong>    <strong>Total</strong></td>
</tr>
<tr>
<td>Neg   106    16    122</td>
</tr>
<tr>
<td>Pos  38    84    122</td>
</tr>
<tr>
<td>Total 144 100 244</td>
</tr>
</tbody>
</table>

McNemars test (p = 0.003)

### 3.4 DISCUSSION

Agreement (78%) was less than had been hoped but is consistent with previous studies comparing the detection of GBV-C RNA between laboratories [Bogard et al., 1997; Kunkel et al., 1998; Lefrere et al., 2000]. Of most concern for UOI were the 3 CDC standards incorrectly identified by UOI and of concern for CDC were the 31 UOI specimens identified as positive by UOI and negative by CDC.

To further examine the sensitivity of the CDC assay, the 31 specimens that tested positive by UOI and negative by CDC were retested by both laboratories. UOI found that 5 of these were, in fact, negative. CDC, on retesting, found that tripling the amount of
RNA (compared to the amount normally used) resulted in 3 additional specimens testing positive for a low level of virus (less than 1000 copies per reaction). Retesting with nested primers resulted in an additional 6 specimens testing positive, one of which had also tested positive above (viral load information not available for nested PCR). Iowa’s methods (nested primers and multiple primers) may be more sensitive than the single primer used by CDC but nested PCR may also yield false positives through the amplification of previously amplified product.

3.5 IMPLICATIONS

The CDC extracted RNA specimens had highly concordant test results between laboratories (91%, 81/89 excluding the 10 plasma standards). Extracted RNA is already partially processed so provides less opportunity for contamination and misclassification. Of the 10 CDC plasma standards, 3 were incorrectly identified by UOI, indicating that at least some of the observed discordance was due to problems with UOI’s specimen classification.

Since CDC is genotyping all RNA positive specimens for our epidemiologic study, we have a method of confirmation and can be less concerned with specificity (false positives). GBV-C RNA positive specimens with a viral load of less than 1,000 copies per reaction where genotyping is unsuccessful may be false positives and will therefore be removed from the analysis, preventing misclassification.
It is possible, though, that through primer mismatch (where the primer region does not match every single base pair due to a slight mutation of the virus), we may miss some GBV-C positive specimens. This research assay has a lower limit of reliable detection of 1,000 copies/reaction (20uL of plasma) similar to other studies. As we are using the single best primer available (the 5’ region contains several well conserved sequences) [Kunkel et al., 1998], we are confident that we are identifying most specimens with a viral load of greater than 1,000 copies per reaction. Results of this validation study indicate that sensitivity of our test may range from 69% to 91%. In case any misclassification does occur, analysis of our data will include a sensitivity analysis to estimate potential bias in any measures of association.

Our collaborators at the University of Iowa published a paper in 2006 comparing the sensitivity and specificity of different primers [Souza et al., 2006]. The 5’UTR primer, the one used for our study, was the most sensitive (100%) of 5 primers tested in nested PCR and specificity was 90%. They recommended using the 5’ UTR primer to screen for GBV-C and then another more specific primer to confirm presence of GBV-C. In our study, we used unnested RT-PCR which is slightly less sensitive than nested PCR and genotype testing to confirm presence of GBV-C in maternal samples.
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APPENDIX 4

ADDITIONAL ANALYSES

Table A4.1: MTCT of HIV by maternal and infant GBV-C status

<table>
<thead>
<tr>
<th>Maternal GBV-C status</th>
<th>Infant GBV-C status</th>
<th>Peri-1</th>
<th>Peri-2 AZT</th>
<th>Peri-2 placebo</th>
<th>Peri-3</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA+</td>
<td>All</td>
<td>16/81</td>
<td>3/31</td>
<td>7/23</td>
<td>5/110</td>
<td>31/245</td>
</tr>
<tr>
<td></td>
<td>GBV-C RNA+</td>
<td>0/21</td>
<td>1/22</td>
<td>0/7</td>
<td>1/51</td>
<td>2/101</td>
</tr>
<tr>
<td></td>
<td>GBV-C RNA-</td>
<td>16/60</td>
<td>2/9</td>
<td>7/16</td>
<td>4/59</td>
<td>29/144</td>
</tr>
<tr>
<td>Neg</td>
<td>GBV-C RNA-*</td>
<td>41/149</td>
<td>12/135</td>
<td>24/141</td>
<td>34/452</td>
<td>111/877</td>
</tr>
</tbody>
</table>

*assumed

Table A4.2 GBV-C viral load measurement among HIV-infected (plasma specimens) and HIV-uninfected women (serum specimens) in Peri-1

<table>
<thead>
<tr>
<th></th>
<th>HIV+ (n=93) plasma</th>
<th>HIV- (n=24) serum</th>
<th>Wilcoxon rank sum test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median GBV viral load</td>
<td>275,000</td>
<td>4,250,000</td>
<td></td>
</tr>
<tr>
<td>Median log10 GBV viral load</td>
<td>5.43</td>
<td>6.58</td>
<td>p=0.03</td>
</tr>
</tbody>
</table>
Table A4.3  Sensitivity analysis of 40 missing infant specimens on association of infant GBV-C with MTCT of HIV

40 missing infant specimens: 11 HIV+, 14 HIV-, 15 HIV status unknown

Assumptions:
- seven (44%) of HIV status unknown are HIV+ (used same proportion as HIV status known even though only 13% of infants in study were HIV-infected)
- 41% MTCT of GBV-C of all specimens (even though only 6.5% of HIV-infected infants were GBV-C-infected)

Missing specimen classification

<table>
<thead>
<tr>
<th>Infant</th>
<th>HIV+</th>
<th>HIV-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBV+</td>
<td>7</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>GBV-</td>
<td>11</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>22</td>
<td>40</td>
</tr>
</tbody>
</table>

Original results

<table>
<thead>
<tr>
<th>Infant</th>
<th>HIV+</th>
<th>HIV-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBV+</td>
<td>2</td>
<td>99</td>
<td>101</td>
</tr>
<tr>
<td>GBV-</td>
<td>162</td>
<td>1070</td>
<td>1232</td>
</tr>
<tr>
<td>Total</td>
<td>164</td>
<td>1169</td>
<td>1333</td>
</tr>
</tbody>
</table>

Sensitivity results (Original results + missing specimens)

<table>
<thead>
<tr>
<th>Infant</th>
<th>HIV+</th>
<th>HIV-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBV+</td>
<td>9</td>
<td>108</td>
<td>117</td>
</tr>
<tr>
<td>GBV-</td>
<td>173</td>
<td>1083</td>
<td>1256</td>
</tr>
<tr>
<td>Total</td>
<td>182</td>
<td>1191</td>
<td>1373</td>
</tr>
</tbody>
</table>

OR 0.52 95% CI 0.26-1.05